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ROR1-a Novel Receptor Tyrosine Kinase with Unique Therapeutic Potentials in Chronic Lymphocytic Leukemia

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of small B lymphocytes in blood, bone marrow, lymph nodes and other lymphoid tissues. CLL is the most common leukemia in the Western world. Despite significant advances in understanding the pathogenesis, CLL is still a disease with no available cure.

Receptor tyrosine kinases (RTKs) are a large family of cell surface receptors participating in crucial cellular processes including proliferation, differentiation, cell-cell interaction, metabolism, signaling, migration and cell survival. More than half of the RTK families are overexpressed or mutated in different forms of human cancer. RTKs are multifunctional therapeutic targets and novel RTKs in cancer have been pursued extensively as a goal for targeted therapies. ROR1 belongs to one of twenty families of RTKs. It is a survival kinase and acts as a receptor for Wnt5a protein.

Gene expression profiling studies have shown that ROR1 was upregulated in CLL patients. Characterization of ROR1 expression in CLL and the study of its functional role for possible therapeutic targeting was the driving force of this thesis.

In the first study we investigated the expression pattern of ROR1 in CLL. All CLL patients (n=18) expressed ROR1 both at gene and protein levels but none of the healthy donors. CLL patients showed a ROR1 surface expression in the range of 36–92%. Western blot analysis revealed two ROR1 bands of 105 and 130 kDa. Mutation analysis of the ROR1 gene showed no major genomic aberrations. FISH analysis of PBMC from 3 CLL patients showed no rearrangement in the 1p region.

The second study was conducted to examine the effects of siRNAs specifically silencing ROR1 and fibromodulin (FMOD) in CLL cells. siRNA treatment induced a specific reduction (75–95%) in FMOD and ROR1 mRNA expression. Western blot analysis for ROR1 and FMOD demonstrated that the proteins were significantly downregulated 48 h after siRNA treatment. Silencing of FMOD and ROR1 resulted in a statistically significant apoptosis of CLL cells but not of B cells from normal donors.

In the third study, five ROR1 monoclonal antibodies were raised against extracellular domains of ROR1 to investigate the in vitro apoptotic effects on CLL cells. All five mAbs induced apoptosis of CLL but not of normal B cells in the absence of complement or immune effector cells. Most effective were mAbs against CRD and KNG, being superior to rituximab in vitro. Cross-linking of the anti-ROR1 mAbs using F(ab´)2 fragments of anti-Fc antibodies significantly augmented apoptosis. Two of the mAbs induced complement-dependent cytotoxicity similar to that of rituximab.

The fourth study was aimed at investigating ROR1 and ROR2 expression in hematological malignancies of lymphoid and myeloid origins. The results showed a statistically significant variation in the expression of ROR1 in various hematological malignancies. No expression of ROR2 was detected in hematological malignancies tested and PBMC of healthy donors. A statistically significant higher expression of ROR1 was detected in progressive compared to non-progressive CLL patients. ROR1 expression was shown to be stable overtime.

In conclusion ROR1 was found to be ectopically expressed in CLL. Given the successful history of RTKs targeted therapies in cancer, ROR1 might be a novel potential therapeutic target structure in CLL.

LIST OF PUBLICATIONS

I. **Daneshmanesh AH**, Mikaelsson E, Jeddi-Tehrani M, Bayat AA, Ghods R, Ostadkarampour M, Akhondi M, Lagercrantz S, Larsson C, Österborg A, Shokri F, Mellstedt H, and Rabbani H. ROR1, a cell surface receptor tyrosine kinase is expressed in chronic lymphocytic leukemia and may serve as a putative target for therapy. *International Journal of Cancer, 123(5):1190-95, 2008*.

II. Choudhury A, Derkow K, **Daneshmanesh AH**, Mikaelsson E, Kiaii S, Kokhaei P, Österborg A, and Mellstedt H. Silencing of ROR1 and FMOD with siRNA results in apoptosis of CLL cells. *British Journal of Haematology, 151(4):327-35, 2010*.

III. **Daneshmanesh AH**, Khan AS, Hojjat-Farsangi M, Jeddi-Tehrani M, Akhondi M, Ali Bayat A, Ghods R, Mahmoudi AR, Hadavi R, Österborg A, Shokri F, Rabbani H, and Mellstedt H. Monoclonal antibodies against ROR1 induce apoptosis of chronic lymphocytic leukemia (CLL) cells. (*Submitted*)

IV. **Daneshmanesh AH**, Porwit-MacDonald A, Hojjat-Farsangi M, Jeddi-Tehrani M, Pokrovskaja Tamm K, Grandér D, Lehmann S, Shokri F, Rabbani H, Österborg A, and Mellstedt H. The receptor tyrosine kinases ROR1 and ROR2 expression in hematologic malignancies. (*Manuscript*)

Related Publications:

A. Mikaelsson E, **Danesh-Manesh AH**, Lüppert A, Jeddi-Tehrani M, Rezvany MR, Sharifian RA, Safaie R, Roohi A, Österborg A, Shokri F, Mellstedt H, and Rabbani H. Fibromodulin, an extracellular matrix protein: characterization of its unique gene and protein expression in B-cell chronic lymphocytic leukemia and mantle cell lymphoma. *Blood, 105(12):4828-35, 2005*.

B. Rabbani H, Ostadkarampour M, **Danesh Manesh AH**, Basiri A, Jeddi-Tehrani M, Forouzesh F. Expression of ROR1 in patients with renal cancer – a potential diagnostic marker. *Iranian Biomedical Journal, 14(3):77-82, 2010*.

LIST OF ABBREVIATIONS

ABC	Antibody binding capacity
ADCC	Antibody-dependent cell-mediated cytotoxicity
AICDA	Activation-induced cytidine deaminase
Ag	Antigen
ALL	Acute lymphocytic leukemia
AML	Acute myelogenous leukemia
APC	Antigen presenting cell
ATM	Ataxia telangiectasia-mutated
β2-M	β2-Microglobulin
BCR	B cell receptor
BCL-2	B cell lymphoma 2
BDB	Brachydactyly B
BM	Bone marrow
B-NHL	B-cell non-Hodgkin lymphomas
CAP	Cyclophosphamide, adriamycine, prednisone
CAR	Chimeric antigen receptor
CDC	Complement-dependent cytotoxicity
CHO	Chinese hamster ovary
CLL	Chronic lymphocytic leukemia
CD40L	CD40 ligand
CDR	Complementarity-determining region
CHOP	Cyclophosphamide, adriamycine, vincrinstine, prednisone
CML	Chronic myelogenous leukemia
CMV	Cytomegalovirus
CNS	Central nervous system
CpG	Cytosine-phosphoguanine dinucleotides
CR	Complete response
CRD	Cysteine-rich domain
DAPK-1	Death-associated protein kinase 1
DLBCL	Diffuse large B-cell lymphoma
ECD	Extracellular domain
EGCG	Epigallocatechin-3-gallate
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
FA	Fludarabine/alemtuzumab
FC	Fludarabine/cyclophosphamide
FCA	Fludarabine/cyclophosphamide/alemtuzumab
FCR	Fludarabine/cyclophosphamide/rituximab
FDA	Food and Drug Administration
FGFR	Fibroblast growth factor receptor
FISH	Fluorescence in situ hybridization
FLT3	Fms-like tyrosine kinase 3
FL	Follicular lymphoma
FMOD	Fibromodulin

FR	Fludarabine/rituximab
GAS	Gamma interferon activation site
GC	Germinal center
GIST	Gastrointestinal stromal tumours
HCL	Hairy cell leukemia
HEK293	Human embryonic kidney
HER2	Human epidermal growth factor receptor 2
HPV	Human papillomavirus
IC	Immunocytoma
ICD	Intracellular domain
IFN	Interferon
Ig	Immunoglobulin
IGFR	Insulin growth factor receptor
IGHV	Ig heavy chain V
IL	Interleukin
IWCLL	International WORKSHOP on chronic lymphocytic leukemia
KNG	Kringle domain
LDH	Lactate dehydrogenase
LDT	Lymphocyte doubling time
LN	Lymph nodes
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MBL	Monoclonal B lymphocytosis
MCL	Mantle cell lymphoma
MCL-1	Myeloid leukemia cell differentiation protein
MFI	Mean florescence intensity
MM	Multiple myeloma
MRCC	Metastatic renal cell carcinoma
MuSK	Muscle-specific kinase
MZL	Marginal zone lymphoma
NF-kB	Nuclear factor kappa B
NGF	Nerve growth factor
NK cell	Natural killer cell
NLC	Nurse-like cells
NP	Non-progressive
NSCLC	Non-small-cell lung cancer
NTRKR	Neurotrophic tyrosine kinase receptor
OR	Overall response
OS	Overall survival
PARP	Poly (ADP ribose) polymerase
PB	Peripheral blood
PC	Proliferation center
PDGFR	Platelet-derived growth factor receptor
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinases
PRD	Prolin-rich domain
PTB	Phosphotyrosine-binding
PTM	Posttranslational modifications

ROR	Receptor tyrosine kinase-like orphan receptor
ROS	Reactive oxygen species
RRS	Recessive Robinow syndrome
RTK	Receptor tyrosine kinase
SHM	Somatic hypermutation
SH2	Src homology 2
SLL	Small lymphocytic lymphoma
SLVL	Splenic lymphoma with villous lymphocytes
SCT	Stem cell transplantation
sIg	Surface immunoglobulin
SNPs	Single nucleotide polymorphisms
SRP	Signal recognition particle
sTK	Serum thymidine kinase
S/TRD	Serine/threonine-rich
TAA	Tumor-associated antigen
TCL1	T cell leukemia/ lymphoma 1
TCR	T cell receptor
TD	T cell-dependent
TGF	Tumor growth factor
TI	T cell-independent
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
TRM	Transplant-related mortality
TNF	Tumor necrosis factor
t-ROR1	Truncated ROR1
VEGFR	Vascular endothelial growth factor receptor
XIAP	X-linked inhibitor of apoptosis protein
ZAP70	Tyrosine kinase zeta-associated protein 70

CONTENTS

1 CHRONIC LYMPHOCYTIC LEUKEMIA

1.1 DEFINITION

Chronic lymphocytic leukemia (CLL) is a clonal disease of unkown etiology and is characterized by the accumulation of small B lymphocytes with mature appearance in blood, bone marrow, lymph nodes and other lymphoid tissues [1]. The B cells in CLL express CD19, CD5, and CD23 and also show decreased levels of membrane IgM, IgD, and CD79b [2-5]. CLL is known to be a heterogeneous disease and follows an extremely variable clinical course with overall survival time after diagnosis ranging from months to decades [6, 7]. CLL may stay non-progressive (indolent) for many years without any treatment required but there are many patients who will encounter the progressive stage of the disease and require treatment for symptoms such as fatigue, disease-related fever, lymphadenopathy, splenomegaly and/or bone marrow failure [8]. Despite significant advances in understanding the pathogenesis of CLL through years and efficient treatment regimens resulting in improved overall survival rates, CLL is still ranked as a disease with no available cure.

1.2 INCIDENCE AND EPIDEMIOLOGY

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the Western world, affecting mainly elderly and accounting for 25-30% of all leukemias [9, 10]. The incidence is about 2–6 new cases per 100,000 individuals per year [9, 11]. In Sweden there are about 500 new cases diagnosed each year [12]. Men are affected by CLL more often than women, with a ratio of about 1.5-2:1 [9, 10]. The disease is twice as common in males as females. CLL is rarer among Blacks, compared to the Whites and much rarer in Asian/Pacific Islanders (75% and 23% that of Whites respectively) [13]. The median age at diagnosis in men is 70 years and in women 74 years [14, 15]. About one third of patients are diagnosed before 55 years of age [9, 16].

1.3 ETIOLOGY AND RISK FACTORS

The etiology of CLL is unclear. Environmental factors appear not to be a major factor in the pathogenesis of CLL [17]. It appears that CLL pathogenesis is related to a genetic and familial predisposition and supported by two series of facts. Firstly, CLL is very rare in China, Korea and basically absent in Japan. Japanese emigrants maintain the low incidence rate within their progeny, thereby excluding an environmental modifier of such genetic predisposition [9, 18, 19]. Secondly, there are reports showing a correlation between CLL development and a family history of the malignancy [15, 20, 21]. Epidemiological studies show that in 5–10% of cases there is a familial susceptibility to CLL with two or more individuals affected in the same family [9, 20, 22, 23]. In fact the first relatives of CLL patients have an overall risk of 2 to 7 times higher than the general population [9, 24]. In a report from the National Cancer Institute Familial Registry, CLL

familial cases were diagnosed at earlier years (mean age diagnosis at 58 years) than sporadic cases [15, 25]. There is no difference in survival from diagnosis in familial compared with nonfamilial cases of CLL [15, 26]. This familial predisposition is accompanied by the genetic anticipation phenomenon, the process in which there is an earlier onset and a more severe course of the disease in the offspring of CLL patients [20, 27-29]. Except for the presentation at earlier age, familial CLL share a high similarity with the sporadic CLL cases in clinical, molecular and biological features.

1.4 DIAGNOSIS

1.4.1 Diagnostic criteria

According to the criteria outlined by the International Workshop on CLL (iwCLL), CLL can be diagnosed when the following conditions exist: A) The presence of at least 5×10^9 B lymphocytes/L (5000/μL) in the peripheral blood for a minimum period of 3 months [30]. Leukemia cells found in the blood smear are characteristically small, mature lymphocytes and can be found mixed with larger or atypical cells or prolymphocytes, these cells may comprise up to 55% of the blood lymphocytes [31]. B) CLL cells coexpress the T-cell antigen CD5 and B-cell antigens CD19, CD20, and CD23. The expression levels of surface immunoglobulins (sIg) including IgM and IgD , CD20, and CD79b are characteristically low compared with those found on normal B cells [5, 32]. As shown in this thesis, also ROR1 may be added to the list of markers that distinguish CLL cells from normal B cells. Each clone of leukemia cells is restricted to expression of either kappa or lambda immunoglobulin light chains [5, 30]. The diagnostic criteria for CLL is summarized in Table 1. A bone marrow aspirate or biopsy is not required at diagnosis in CLL [33]. In contrast bone marrow examination is part of the complete remission state, as absence of leukemic involvement of the bone marrow is required for this state.

Table 1. Diagnosis criteria for CLL.

- Presence of ≥5,000 clonal B lymphocytes in the peripheral blood
- Peripheral blood smear: small, morphologically mature lymphocytes with a narrow border to cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin, <55% prolymphocytes, Gumprecht nuclear shadows or smudge cells
- Immunophenotype: coexpression of the T-cell antigen CD5 and B-cell surface antigens CD19, CD20 and CD23, levels of CD20 and CD79b are low compared with normal B cells, expression of either kappa or lambda immunoglobulin light chains

Adapted from Cramer P, Hallek M, 2011 [34]

1.4.2 Differential diagnosis

Continued elevation of the lymphocyte count demands for a differential diagnosis among other lymphoproliferative disorders. The characteristic features discussed above for CLL,

differentiate it from mantle cell lymphoma (MCL), hairy cell leukemia (HCL), follicular lymphoma (FL) and marginal zone lymphoma (MZL), in particular splenic lymphoma with villous lymphocytes (SLVL) (Figure 1). In fact MCL also expresses CD5 but is negative for the expression of CD23 and it frequently carries the t(11;14) translocation involving the cyclin D1 gene [9]. The other malignancies mentioned are CD5 negative and carry a distinct phenotype as detected by flow cytometry [4, 5]. In a disorder very similar to CLL, known as small lymphocytic lymphoma (SLL), the tumor is restricted to lymph nodes or other tissues without blood involvement. SLL has identical histological and immunophenotypic findings to CLL and management of these two malignancies is the same [7, 35]. It has been shown that CD5+ monoclonal B cells carrying the distinct phenotype of CLL are present in about 3.5% of healthy individuals with normal blood counts and can be recognized by flow cytometry [9, 36, 37]. This situation has been named monoclonal B cell lymphocytosis (MBL), is age-dependent (seen in >7% of individuals over 70 years) and more common in immediate relatives of CLL patients [38, 39].

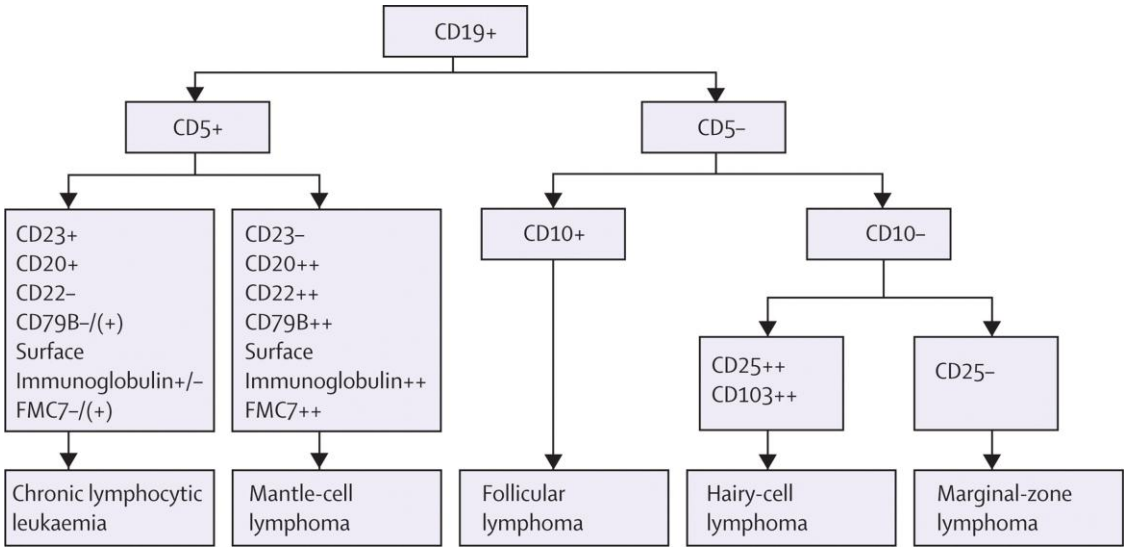

Figure 1. Differential diagnosis of CD19+ lymphocytosis.
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1.5 STAGING

Two staging systems are presently used to describe the extension and prognosis of CLL, the Rai system [40] and the Binet system [41]. These two methods can also be used to estimate the starting time of CLL therapy. Originally the number of prognostic groups in the Rai classification was 5 but later it was modified to 3 prognostic groups [42] and now both these systems describe 3 major subgroups with different clinical outcomes. These two staging methods are simple, cheap and are applicable worldwide. Both are dependent simply on physical examination and standard laboratory tests and do not require advanced diagnostic medical equipments. The Rai and the Binet staging systems are summarized in Table 2.

Table 2. Rai and Binet clinical staging systems for CLL.

System	Clinical features	Median survival, year
Rai stage (simplified 3-stage)		
0 (low risk)	Lymphocytosis in blood and bone marrow	>10
I and II (intermediate risk)	Lymphadenopathy, splenomegaly +/- hepatomegaly	7
III and IV (high risk)	Anemia, thrombocytopenia	0.75 – 4
Binet group		
A	Fewer than 3 areas of lymphadenopathy; no anemia or thrombocytopenia	12
B	More than 3 involved node areas; no anemia or thrombocytopenia	7
C	Hemoglobin <100 g/L, platelets <100 x10 g/L	2-4

Adapted from Gribben JG, Blood, 2010 [15]

1.6 PROGNOSIS

The natural course of CLL is extremely heterogeneous with overall survival time stretching from months to decades with a median of about 7.5 years [43]. In contrast to other hematological malignancies, age per se is not a prognostic factor due to the fact that the biological features and the clinical course of the disease is identical in both young (<55 years) and old patients with the overall expectancy of life about 10 years in both groups [9]. Prognostic factors efficient in predicting the course of CLL at the initial stages of diagnosis are becoming critical for the proper management of CLL patients. Prognosis of CLL is dependent on two sets of factors, clinical and biological prognostic factors (Table 3 and Figure 2).

Table 3. Prognostic factors in CLL that predict an unfavorable outcome

Traditional markers
Advanced Rai and Binet clinical stage
Lymphocytic doubling time <12 months
Elevated lactic dehydrogenase
Elevated soluble CD23
Elevated thymidine kinase
Elevated β-2 microglobulin
Diffuse pattern of bone marrow infiltration
Newer markers
del(11q) and del(17p) using interphase fluorescent *in situ* hybridization (FISH)
TP53 mutation (with or withour 17p-)
Unmutated immunoglobulin heavy chain variable region genes (IGVH genes)
VH3.21 gene usage independent of IGVH
ZAP-70 (≥ 20% positive)
CD38 (≥ 30% positive)

Adapted from Foon KA, Hallek MJ, Leukemia, 2010 [6]

1.6.1 Clinical prognostic factors

The first prognostic markers to supplement the clinical staging methods were based on the morphology of CLL cells in blood and bone marrow. The clinical stage of CLL diagnosed according to the Rai or the Binet staging systems can be helpful in prognosis of the disease. A better prognosis is seen in Rai stage 0 (low risk) and Binet stage A compared to the other stages [33]. If the lymphocyte doubling time (LDT) is less than 12 months it indicates disease progression [44], i.e. decreased progression-free and overall survival time [45, 46]. A diffuse bone marrow infiltration pattern correlates with a poorer prognosis [47, 48], the relevance of this factor to clinical practice has diminished with finding new potent prognostic factors. These prognostic factors seem to be incapable of predicting the progression of the disease at initial diagnosis.

1.6.2 Biological prognostic factors

Several biological prognostic factors have been identified that in association with the clinical parameters may help to better define the patient's prognosis. Extensive research resulted in better understanding of the pathogenesis and led to the development of new biological markers that predict the prognosis of CLL patients. Several additional markers of potential prognostic value have been recently described, such as vitamin D deficiency, NK cell G2D receptors/ligands, angiopoetin-2, mcl-1 as well as SNPs. The space and focus of this thesis does however not allow to discuss them in detail. Some traditional and also some of verified/confirmed biological prognostic factors are discussed below.

1.6.2.1 Serum markers

The most important serum markers in CLL patients are serum β2-microglobulin (sβ2m), serum thymidine kinase (sTK), and soluble CD23 (sCD23) and are considered among the traditional biologic factor for prognosis. β2m is an extracellular protein that is bound noncovalently to the α chain of the class I MHC. In CLL, elevated sβ2m levels correlate with advanced disease stage, high tumor burden and shorter progression-free (PFS) and overall survival [49-51]. Serum thymidine kinase is involved in a DNA synthesis pathway, is found in dividing cells and can be measured by radioassay [52]. High levels of this enzyme in CLL patients serum is associated with disease progression and advanced stage disease [53, 54]. CD23 is a low-affinity receptor for IgE, expressed on mature B cells, antigen-presenting cells and platelets. Soluble CD23 serum levels (sCD23) inversely correlate with overall survival and disease progression in Binet stage A patients [55, 56]. Contrary to other lymphoid malignancies, the level of serum lactate dehydrogenase has become less relevant in CLL patients [34, 57] but still there are reports showing elevated lactate dehydrogenase (LDH) serum level is associated with shorter survival time for CLL patients [58, 59].

1.6.2.2 IGHV mutational status

The presence or the absence of somatic mutations within the IGHV genes correlate with clinical stage of CLL patients and carry an independent prognostic value in CLL. This mutation is discussed in detail below (see 1.7.1). A similarity of more than or equal to 98% of the IGHV gene with germline sequence, i.e. unmutated cases, is associated with the aggressive stage of the disease and a shorter median survival time (8 years vs 24 years) as compared to mutated cases (homology <98%) [60, 61]. It is worth mentioning that according to the Swedish CLL guidelines, the influence of IGHV mutational status on clinical management of patients is not to the same extent as cytogenetics (http://www.swecll.org/Nationella-riktlinjer).

1.6.2.3 Surrogate markers

The sequencing of immunoglobulin genes and determination of their mutation status is difficult and expensive. The search for parameters that are strongly correlated with IGHV mutation is ongoing. CD38 and ZAP70 expressions are among the known surrogate markers reported for CLL. CD38 is a surface protein expressed in early B cell development and plasma cells [62]. Expression of CD38 in CLL is associated with unmutated IGHV and correlated with poor prognosis [60, 63, 64]. Despite all these reports , there are still points to be considered, such as no consensus about the best cut-off value is reached and also debate exists on the fact that CD38 expression may vary over time especially after the initiation of therapy [65, 66]. The 70-kD zeta-associated protein (ZAP70) is a signaling protein in the T cell receptor complex, expressed on CLL cells but not on normal B lymphocytes and is also thought to have a role in signaling through the B cell receptor in CLL cells [67, 68]. Several studies have shown that mutated cases in CLL are mostly ZAP70-negative and unmutated cases ZAP70-positive demonstrating an aggressive form of the disease and a poor prognosis [67, 69, 70]. In contrast to CD38, ZAP70 expression appears to be stable over time. There are reports showing that no absolute connection exists between ZAP70 expression and IGHV mutation, with discrepancies occurring in up to 25% of CLL cases [71, 72]. The expression of ZAP70 appears to be a useful predictor of the time to first treatment when compared with the expression of CD38 or IGHV mutation status [71, 73].

1.6.2.4 Chromosomal abnormalities

Fluorescence in situ hybridization (FISH) is the most useful clinical method for chromosomal analysis in CLL and has significant prognostic importance (see also section 1.7.2). Deletion of 13q14, deletion of 11q22, trisomy 12, deletion of 17p13 and deletion of 6q21 are the most frequent chromosomal abnormalities reported in CLL [74, 75]. Deletion of 17q13 results in loss of p53 and is associated with poor response to therapy, rapid disease progression and short survival [75, 76]. Deletion of 11q23, resulting in loss of the ATM gene, is associated with more aggressive form of the disease and poor prognosis [77, 78]. Trisomy 12 does not significantly affect survival as compared to

normal karyotype and patients with deletion 13q14 only, have the most favorable prognosis [75, 77].

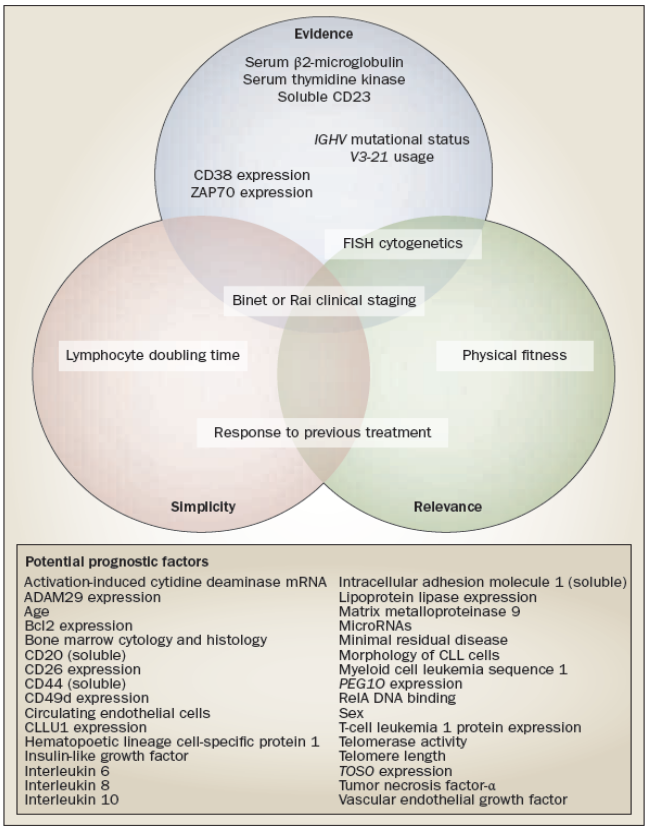

Figure 2. Proposal for the use of prognostic factors in clinical practice based on the three criteria evidence, simplicity and relevance

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1.7 PATHOLOGY AND BIOLOGY

Most CLL cells are quiescent and arrested in G0/G1 of the cell cycle and there is only a small proliferative compartment. CLL is viewed as two similar entities, mutated and unmutated, both of which originate from antigen-stimulated mature B lymphocytes. These entities either avoid death through the intervention of external signals or die by apoptosis, only to be replenished by proliferating precursor cells. Pathogenic mechanisms in CLL involve two kinds of events: external events such as stimuli from the microenvironment and antigenic drive and internal events like genetic and epigenetic processes. These mechanisms are found to be crucial in the transformation, progression and evolution of CLL.

1.7.1 Normal B cell encounter, BCR response and IGHV mutation

The interaction of normal mature B cells with their antigens occur in a microenvironment characterized by a network of cells, molecules and signals and is mediated through the B cell receptor (BCR). The antigen encounter triggers the processes of B cell activation, proliferation and differentiation that result in the generation of antigen-specific memory B cells and plasma cells [79, 80]. The same mechanism of response to antigenic

stimulation is seen in malignant B cells. Each B cell displays a distinct BCR that is formed through variable combinations of V, D and J segments for the Ig heavy chain and V and J gene segments for the light chain. In addition to the combinatorial diversity of different segments, the BCR repertoire is increased by the introduction of somatic mutations through the somatic hypermutation (SHM) process during the germinal centre (GC) reaction (reviewed in [81]) [1]. SHM is an affinity-maturation mechanism that turns a naive B cell expressing low-affinity surface Ig (sIg) to a long-lived, recirculating memory B cell that is a high-affinity antibody producer [79, 82]. As mature B lymphocytes, CLL cells express BCR on the cell surface, which is encoded by the immunoglobulin (Ig) genes. Low expression of the BCR is characteristic of CLL lymphocytes [83]. The reason why there is a poor expression of the BCR in CLL remains obscure. Except for one report of mutation in CD79B [84], no genetic defects in BCR components have been recorded [85, 86].

The discovery that 50% of CLL cases have undergone somatic mutation in IGHV gene was a major advance in the understanding of CLL [87, 88]. Studies revealed that CLL patients can be divided into either unmutated or mutated groups based on the presence or the absence of IGHV gene mutations in CLL cells. Unmutated cases are defined as those having ≥ 98% similarity of the IGHV gene with the germline sequence while those having < 98% homology are defined as mutated. It was also demonstrated that the IGHV mutational status is of prognostic significance. In fact, mutated cases have a more indolent clinical course and longer survival than the unmutated cases [60, 61]. The IGHV mutational status also correlates with a few of other biological prognostic factors such as ZAP70 and CD38 expression. Studies have revealed that patients with unmutated IGHV genes exhibit a higher expression of ZAP70 and CD38, have shorter telomeres and are genetically unstable as compared to mutated cases [60, 74, 89-91]. The activation-induced cytidine deaminase (AICDA), a B cell-restricted enzyme required for somatic mutation and isotype switching, is also upregulated in unmutated CLL cases [92-94].

Somatic mutation of IGHV genes does not occur uniformly. Some Ig gene segments such as IGHV1-69, for example, consistently carry few mutations as opposed to the typically mutated IGHV3-7, IGHV3-23, and IGHV4-34 genes.

There are structural differences of the BCR between IGHV-mutated and unmutated CLLs. IGHV-unmutated CLL cases more frequently show stereotyped rearrangements of the V, D and J segments that have very similar complementarity-determining region (CDR) 3 regions and display stereotyped light chains and biased somatic mutation patterns [95-97]. Studies have demonstrated that more than 20% of CLL cases carry stereotyped BCRs and in 1% of cases the Igs are nearly identical, suggesting that common antigens are recognized in many patients with CLL [98-102]. The observation that a stereotyped BCR, such as IGHV3-21, is associated with worse prognosis even if mutated indicates a possible role of an unknown common antigen, but still the role of antigen drive in CLL pathogenesis has to be clarified [103]. Extensive work is underway trying to define these antigens, which may be autoAg or exogenous [104-106] and a picture of CLL as an antigen-driven or partly autoimmune disease is emerging. Some of the antigens currently discussed in CLL are cytoskeletal proteins vimentin, filamin B and cofilin-1, along with phosphorylcholine-

containing antigens (eg. Streptococcus pneumonia polysaccharides), cardiolipin and proline-rich acidic protein-1 [107].

Taken together, although the cellular origin of CLL cells has not been finally clarified, there is strong evidence that IGHV-mutated CLLs (mostly) stem from antigen-experienced post-GC memory B cells [1]. However, the possibility that the IGHV mutated cells might derive from B cells that accumulate SHM in a T cell-independent (TI) immune response, that does not involve the GC, or during a primary, Ag-independent BCR diversification process should also be considered [108]. IGHV-unmutated CLLs also originate from antigen experienced B cells that acquire features of memory B cells. Whether these are activated conventional naive B cells, CD5+ B cells or marginal zone-like B cells is not clear. Either this activation takes place as part of a TI or T cell-dependent (TD) immune response is also yet to be understood [1].

1.7.2 Genomic aberrations and gene mutations

Contrary to other low-grade B-cell malignancies, translocations are rarely seen in CLL. However aberrations resulting in loss or gain of genetic material, appear during the course of the disease and are of prognostic significance [9]. The use of fluorescence in situ hybridization (FISH) technique has allowed the detection of one or more chromosomal abnormalities in more than 80% of CLL patients [75]. The most common chromosomal aberrations detected in CLL are deletions of 13q14 and 11q22–q23, trisomy 12 and deletion of 17p13 [74, 75]. Deletion of 13q14 occurs in more than 50% of CLL cases, making it the most frequent aberration in CLL. Studies have shown this deletion actually involves two noncoding microRNA genes, mir15-a and mir-16-1 [109], which are believed to take part in controlling antiapoptotic genes in leukemic cells [110]. In 10-20% of the cases, 11q22–q23 deletion is seen. The ataxia telangiectasia-mutated (ATM) gene is located within the minimal consensus region of chromosome bands 11q22.3–q23.1 and is affected in almost all cases with del 11q22-q23 [111, 112]. The ATM protein kinase is a central component of the DNA damage pathway and mediates cellular responses to DNA double-strand breaks. Trisomy of chromosome 12 is detected in 10–20% of the cases. Despite being the least common aberration (in less than 10% of the cases), deletion of 17p13 is involved with p53 locus and is associated with rapid progression of disease, poor response to conventional chemotherapy and short survival. It is shown that deletions and mutations in the p53 gene lead to disease progression and alternation of sensitivity to chemotherapy drugs in CLL [75]. There are doubts regarding stability of cytogenetic changes over time in CLL, so it is important to use the whole panel of FISH markers.

1.7.3 Epigenetic alterations

The epigenetic mechanisms involve changes in the gene function of the cell without a change in the DNA sequence. In normal circumstances, epigenetic changes are important for proper development and function of the cell, but in cancer epigenetic aberrations

promote uncontrolled growth of the tumor cells [113] and also result in alteration of gene function and malignant transformation of cells [114]. Studies have shown that DNA methylation leads to transcriptional gene silencing and aberrant DNA methylation plays an important role in tumorigenesis. Like other cancers, altered methylation of DNA (hypomethylation) has been described for several genes specifically deregulated in CLL, such as BCL2 [115] and T cell leukemia/ lymphoma 1 (TCL1) [116]. An analysis of the global methylation profile found that 2.5-8.1% of the CpG (cytosine-phosphoguanine dinucleotides) islands in CLL samples were aberrantly methylated [117]. Such methylation processes are usually associated with stable silencing of the concerned gene and it was suggested that a portion of these methylation events confer a selective advantage to the malignant cells [117].

In CLL patients a correlation between the promoter methylation and transcriptional silencing of certain genes, such as death-associated protein kinase 1(DAPK1) and ZAP70 have been found [118, 119]. Being reversible, epigenetic changes in cancer can be suitable therapeutic targets. Histone deacetylase inhibitors are among several compounds tested on CLL cells in vitro, however in CLL the results have not been convincing enough compared to other leukemias [120].

1.7.4 Microenvironment

It is known that CLL cells show prolonged survival in vivo and accumulate in peripheral blood, bone marrow and lymphoid organs which could be due to their resistance to undergo programmed cell death [2]. The importance of CLL microenvironment is understood by the fact that CLL cells rapidly undergo apoptosis when cultured in vitro under conditions that support the growth of human B cell lines [121]. This indicates that CLL cell resistance to apoptosis depends on external signals rather than being an intrinsic feature of the leukemic cells [2, 122, 123]. Moreover, if cocultured in vitro with bone marrow-derived stromal cells, fibroblasts, dendritic cells, nurse-like cells (NLC) or T cells, CLL cells can be rescued from apoptosis [124-127]. These cells provide a variety of survival stimuli for CLL cells, mediated via soluble factors, extracellular matrix components and signals derived from cell-cell contact [128]. Despite the fact that CLL cells are largely arrested in G0/G1 phase of the cell cycle, there is a small proportion of leukemic cells that continue to divide in the proliferation centres (PC) and interact with prolymphocytes and paraimmunoblasts [129]. Proliferation centers are focal aggregates of variable size found in lymph nodes and to a lesser extent in the bone marrow [130, 131]. At these centers, the growth of leukemic cells is favored by T cell help and also by interaction with stromal cells. The form of support offered by T and stromal cells is different, T cells provide a short-term support while stromal cells provide a long-term support, resulting in prolonged survival of tumor cells and consequently the accumulation of leukemic cells [124, 132, 133]. Studies have demonstrated the presence of an increased number of T cells, mostly being CD4+ T cells that express CD40 ligand (CD40L). The interaction between CD40 and its ligand, CD40L synergizes with BCR signaling and in

turn induces several anti-apoptotic signaling pathways, including survivin and NF-κB [132, 134, 135].

1.8 TREATMENT

Most often CLL patients do not present any symptoms or signs of the disease, they are simply identified because of a blood test requested for an unrelated reason. Most of these patients will not need treatment and never progress, however, those who do need treatment, are usually presented in the same way. A period of watchful waiting until features of progression are observed constitutes the standard management of CLL. The signs of progression include bulky lymphadenopathy and/or splenomegaly, marrow failure or rapid progression of the disease. The criteria for active disease, defined in the International Workshop on CLL (iwCLL) guidelines [30], form the basis for treatment initiation in both clinical trials and in routine practice (Table 4).

Table 4. International workshop on CLL guidelines for the initiation of treatment

Evidence of progressive marrow failure as manifested by the development of, or worsening of, anemia and/or thrombocytopenia.
Massive (i.e., > 6 cm below the left costal margin) or progressive or symptomatic splenomegaly.
Massive nodes (i.e., > 10 cm in longest diameter) or progressive or symptomatic lymphadenopathy.
Progressive lymphocytosis with an increase of more than 50% over a 2-month period or lymphocyte doubling time (LDT) < 6 months.
Autoimmune anemia and/or thrombocytopenia poorly responsive to corticosteroids or other standard therapy.
Constitutional symptoms, defined as any one or more of the following: • unintentional weight loss ≥ 10% in the previous 6 months; • significant fatigue (i.e., Eastern Cooperative Oncology Group (ECOG) PS ≥ 2; inability to work or perform usual activities); • fevers higher 38.0°C for ≥ 2 weeks without other evidence of infection; or • night sweats for ≥ 1 month without evidence of infection

Adapted from Hallek M et al, Blood, 2008 [30]

1.8.1 Cytostatic drugs

The golden standard procedure for first-line treatment in CLL (monotherapy) for several decades has been alkylating agents such as chlorambucil or cyclophosphamide [136]. Up to 70% overall response is reached with chlorambucil alone however complete response (CR) is only less than 10%. Almost all patients relapse over time, their response to salvage therapy is of shorter duration after every relapse and most of them will die from disease-related causes. Chlorambucil is only indicated when palliative purposes is the goal of treatment [9]. Recently bendamustine, a purine analogue/alkylator hybrid agent, was shown to be effective as first line therapy in CLL [137]. Combinations of alkylating agents and anthracyclines such as CAP (cyclophosphamide, doxorubicin, prednisone)

[138] and CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) [139] have shown higher response rates than chlorambucil, but with no survival benefits [9].

In the 1980s, the front-line treatment model for CLL was changed with the introduction of purine-analogues, especially fludarabine. Fludarabine showed to be able to prevent the repair of DNA interstrand crosslinks induced by alkylating agents [140]. Fludarabine has been the most studied and used drug among other purine-analogues (e.g. cladribine and pentostatin). An overall response of 80% and a complete response of 38% have been achieved with single agent fludarabine as first-line therapy [141]. Although fludarabine demonstrated superior overall response compared to chlorambucil or alkylating regimens, no difference in terms of survival could be observed [139, 142].

Studies showed a synergism between fludarabine and cyclophosphamide and this provided a rationale for combining both classes of drugs together [140]. The most widely studied combination chemotherapy regimen for CLL is fludarabine plus cyclophosphamide (FC) [143]. Randomized trials have compared single-agent fludarabine to the FC combination. All these trials demonstrated significantly higher overall response, complete response and progression free survival rates for the combination regimen [144-146], but so far no survival benefit has been observed.

1.8.2 Monoclonal antibodies

Alemtuzumab is a humanized monoclonal antibody (mAb) directed against the CD52 antigen. CD52 expression is detected on most normal and malignant B and T lymphocytes including monocytes and macrophages [147]. Alemtuzumab mechanism of action in CLL is thought to be through antibody-dependent cell-mediated cytotoxicity (ADCC), complement activation and possibly also by direct apoptosis induction [148]. There are many side effects of alemtuzumab due to the widespread expression of CD52, the most common adverse effects are prolonged lymphopenia with an increased risk of infections [9]. In about 20% of CLL patients, cytomegalovirus (CMV) reactivation occurs usually after 3–8 weeks of alemtuzumab therapy and it is recommended that patients should be closely monitored. Alemtuzumab is traditionally given through intravenous (IV) infusion but acute administration related events could be diminished, if alemtuzumab is administrated subcutaneously (SC) without the need of a dose-escalation schedule [149]. At first, the efficacy of alemtuzumab was investigated in CLL patients with refractory/relapsed disease [150-152], these studies demonstrated an overall response rate of 40% in refractory CLL [151, 153] with noticeable effects in CLL patients with deletion of 17p [154]. Encouraging results have also been reported in previously untreated patients. As first-line treatment, alemtuzumab efficacy was shown in a phase II [155] and phase III trials, with phase III trial revealing a significantly higher OR (83% vs 55%), CR (24% vs 2%) and longer PFS compared to chlorambucil [156].

Rituximab is a chimeric murine (mAb) directed against the surface antigen CD20. CD20 expression is seen on both normal and malignant B cells. This antibody is most often used as single agent or combination therapy in relapsed or refractory indolent non-Hodgkin lymphomas. Rituximab exerts its cytotoxic activity through complement-mediated lysis,

ADCC and direct induction of apoptosis. Several studies in previously untreated CLL patients have demonstrated that rituximab as single agent in CLL is inferior to fludarabine [157] and also considerably less effective than in follicular lymphoma [158, 159].

Ofatumumab is a fully human mAb targeting a different epitope on CD20 than the one targeted by rituximab and has been demonstrated to induce higher complement-mediated CLL cell lysis in vitro [160, 161]. In a recent study, an overall response rate of 50% was obtained in refractory CLL [162], leading to approval of ofatumumab by the FDA and by the European Medicines Agency (EMA).

1.8.3 Chemoimmunotherapy

There has been additional success in managing CLL with a combination regimen of cytostatic agents (fludarabine and cyclophosphamide) and monoclonal antibodies. The basis for this combination treatment plan was from preclinical evidence showing synergy between rituximab and chemotherapeutic agents [163-165]. In some initial studies conducted to evaluate the effects of rituximab combined with fludarabine (FR), the results were quite promising [166, 167].

In a randomized phase II trial, the combination of rituximab with fludarabine regimens was investigated and demonstrated a higher significant complete remission rate for the simultaneous administration of fludarabine and rituximab (FR) (47% CR) as compared to (28% CR) of the sequential treatment (F followed by R) [168]. By retrospective comparison, the results of this trial were correlated with a previously conducted study of 178 untreated CLL patients treated with fludarabine alone [142]. This study revealed an advantage in terms of progression-free survival and overall survival given by the FR combination compared to fludarabine alone [169].

In one report, as first-line treatment of CLL patients, the overall response rate of 95% and complete remission rate of 70% were reported in the FCR group. In controls treated with fludarabine and cyclophosphamide (FC), an overall response rate of 88% and complete remission rate of 35% were recorded [170]. A recent data from the first randomized trial, fludarabine and cyclophosphamide (FC) versus fludarabine, cyclophosphamide and rituximab (FCR), showed higher overall response rate, more complete remissions and longer median progression-free survival rate for the FCR treated patients [171]. Particularly, this study was able to demonstrate for the first time that a specific first-line treatment for CLL resulted in a significantly improved overall survival. The percentage of patients alive, in the FCR arm was 87% versus 83% for the FC treated patients, three years after randomization. Of significance, in this trial was the fact that a population of fairly young and physically fit patients were included.

Furthermore, patients with deletion of 17p demonstrated the worst prognosis and no significant difference in overall survival were noted between the two treatment arms. FCR was approved by European Medicines Agency (EMA) as first-line CLL treatment in 2009. In a phase III study, the combination of alemtuzumab with FC was not successful, due to increased toxicity in FCA arm, in seven (7/82) patients the toxicity was fatal [172],

whereas another trial on FCA (using a lower dose of alemtuzumab) has been completed as planned (results pending).

1.8.4 Stem cell transplantation

High complete remissions, including molecular remission, can be achieved by autologous stem cell transplantation in CLL patients, but due to the persistent relapse of the disease, still there is no definitive cure with this form of therapy [173, 174]. In order to find the best strategy regarding stem cell transplantation, there is a need for clinical trials to compare autologous or allogenic hematopoietic stem cell transplantation (SCT) and chemoimmunotherapy regimens.

Moreover, due to the advanced age, indolent clinical course in the majority of CLL patients and a high (20 to 41%) transplant-related mortality (TRM), SCT can not be considered as a treatment option in most cases [175]. European Bone Marrow Transplant guidelines provide indications for SCT in CLL, recommending allogeneic SCT for younger patients who do not respond or relapse early (<12 months) after first-line combination chemotherapy or in patients with p53 abnormalities requiring treatment [176, 177].

1.8.5 Emerging therapies

The past decade has seen major advances in treatment options for CLL, but still CLL remains an incurable disease. At present, the most effective treatment for CLL consists of a combination of fludarabine, cyclophosphamide, and rituximab (FCR). In previously untreated patients FCR combination therapy results in an overall response rates (ORR) of 95%, complete remission (CR) rates of 44% and a median progression-free survival (PFS) of 52 months [178].

Despite all these advances, in addition to successive relapse rates, CLL patients have also become increasingly resistant to treatment. Therefore, new agents with novel mechanisms of action are needed, especially for relapsing CLL patients. Novel mAbs, immunomodulating drugs, Bcl-2 inhibitors, protein kinase inhibitors and small molecule signal transduction inhibitors are among the emerging therapies discussed in this section [179].

Lenalidomide is a derivate to thalidomide and belongs to a class of drugs known as immunomodulating drugs (IMiDs). Lenalidomide antitumor effects are mediated through the inhibition of TNF-alpha and other prosurvival cytokines, T and NK cells activation, as well as antiangiogenic activity. In two phase II trials evaluating lenalidomide for treatment of patients with relapsed or refractory CLL, the rates for OR were 47% and 32%, respectively and CR of 9% and 7%, respectively [180, 181].

There are a number of mAbs in preclinical studies and early phases of clinical trials in CLL (Table 5). Some of these include mAbs targeting CD19, such as the BiTE Ab blinatumomab, CD20 (GA101, veltuzumab, PRO131921), CD80 (galiximab), CD74 (milatuzumab) and CD40 (dacetuzumab).

Lumiliximab is a genetically engineered (macacue variable regions, human constant regions) mAb targeting CD23, a transmembrane glycoprotein, inducing several antiproliferatives functions including apoptosis [179]. In combination with FCR in a phase I/II trial, the OR rate was 65%, with 52% of patients achieving a CR [182].

Obinutuzumab (GA101) is a novel third generation fully humanized mAb. The Fc-region of GA101 is glycoengineered to result in higher affinity binding to the CD20 type II epitope [183]. A phase I study showed that GA101 has a comparable safety profile to rituximab [184]. The mechanism of action of GA101 seems to be through complement and antibody-dependent cell-mediated cytotoxicity [185]. Compared to rituximab GA101 shows an elevated ADCC as well as a markedly higher induction of direct cell death in vitro [186]. GA101 is able to potently elicit actin-dependent, lysosomal cell death in CLL cells in vitro [187]. Trials are now underway to investigate combinations of chlorambucil with novel CD20 antibodies (GA101, ofatumumab) in older CLL patients [188].

In addition, many small molecule inhibitors are under investigation, including Bcl-2 inhibitors, such as oblimersen, obatoclax, AT-101 and ABT-263. Oblimersen alone has shown modest activity but in combination with FC regimen, the overall survival was enhanced compared to FC alone [189].

Protein kinase inhibitors such as flavopiridol have also been studied in CLL therapy. Flavopiridol is a pan-inhibitor of cyclin-dependent kinases and it induces apoptosis by downregulation of the anti-apoptotic protein Mcl-1 in CLL [179]. Sixty four patients with relapsed CLL, all previously treated with purine analogue therapy, were treated with single-agent flavoperidol. The rates for OR and CR were 53% and 2% respectively and median PFS for all patients was 9 months [190].

Phosphatidylinositol 3-Kinases (PI3Ks) are a family of intracellular signaling proteins that are crucial components of proliferative, differentiation and survival pathways in many cells including those of hematopoietic origin. CAL-101 is an inhibitor of the p110δ isoform of PI3K, which has an expression pattern largely restricted to cells of hematopoietic origin. In a phase I study with CAL-101 and 54 CLL patients enrolled, the results showed that the overall response rate was 26% and 80% of CLL patients had a greater than 50% reduction in lymphadenopathy [191].

Autologous T cells can be genetically manipulated to target specific tumor antigens and this constitutes an attractive strategy for cancer therapy [192, 193]. Chimeric antigen receptors (CARs) combine an antigen recognition domain of a specific antibody with an intracellular domain of the CD3-zeta chain or FcγRI protein into a single chimeric protein [194]. The limited in vivo expansion of CAR T cells has been a major obstacle to the clinical application of this technique [192, 193].

The CAR–mediated T-cell responses can be further improved by a costimulatory domain such as inclusion of the CD137 (4-1BB) signaling domain. This significantly increases antitumor activity and in vivo persistence of CARs as compared with the CD3-zeta chain alone [195].

The results of two recent studies show that the engineered T cells expanded more than 1000-fold in vivo, migrated to bone marrow and for at least 6 months continued to express functional CARs at high levels. Toxicity was seen in the form of B cell aplasia in addition

to decreased numbers of plasma cells and hypogammaglobulinemia. Moreover, at least 1000 CLL cells were eradicated by each infused CAR-expressing T cell. Additionally, in the blood and bone marrow a CD19-specific immune response was shown by a potent antileukemic effect in all three patients examined, accompanied by CR in two of three patients with p53-deficient leukemia. Furthermore, a portion of these cells persisted as memory CAR+ T cells and retained anti-CD19 effector functionality [196, 197].

There are reports on therapeutic approaches for two RTKs expressed in CLL, VEGF receptor and Axl RTKs. These targeted therapies along with future role of ROR1 in therapy of CLL is discussed in section 3.5 (targeted therapies of ROR receptor tyrosine kinases).

Table 5. Newer monoclonal antibodies in clinical development in CLL and related B cell disorders

Agent	Target	Humanized/ chimeric	Direct cell death	ADCC	CDC	Development status
MDX-1342	CD19	Humanized	Yes	Yes	No	Phase 1
XmAb5574	CD19	Humanized	Modest	Yes	No	Phase 1
Ofatumumab	CD20	Humanized	Yes	Yes	Yes	Phase 3 (approved)
GA-101	CD20	Humanized	Yes	Yes	Modest	Phase 2
PRO131921	CD20	Humanized	Yes	Yes	Yes	Phase 1/2
Veltuzumab	CD20	Humanized	Yes	Yes	Yes	Phase 1/2
LFB-R603	CD20	Chimeric	Yes	Yes	Yes	Phase 1
Lumiliximab	CD23	Primatized	Yes	Yes	Yes	Phase 3
TRU-016	CD37	Humanized	Yes	Yes	No	Phase 1
SGN40	CD40	Humanized	Yes	Yes	No	Phase 1/2
HCD122	CD40	Humanized	No	Yes	No	Phase 1
MDX-1411	CD70	Humanized	No	Yes	No	Phase 1
Milatuzumab	CD74	Humanized	Yes	No	No	Phase 1/2
Ipilimumab	CTLA-4	Humanized	Yes	No	No	Phase 2
Bevacizumab	VEGF	Humanized	No	No	No	Phase 2

Adapted from Jaglowski SM et al, Blood, 2010 [198]

2 RECEPTOR TYROSINE KINASES

2.1 INTRODUCTION

Receptor tyrosine kinases (RTKs) are a large family of cell surface receptors discovered more than 20 years ago [199]. These receptors participate in crucial cellular processes, such as proliferation, differentiation, cell-cell interaction, metabolism, signaling, migration and cell survival [200, 201]. RTKs are divided into 20 different receptor families consisting of 58 members (Figure 3) [202]. More than half of these receptor families have been reported to be overexpressed or mutated in different forms of human cancer, therefore RTKs are considered as targets for cancer therapy [200].

All RTKs have a similar molecular structure, an extracellular region containing the ligand binding domains, a single transmembrane region and an intracellular region with conserved tyrosine kinase (TK) domain. Ligand binding results in dimerization of the extracellular part and initiation of intracellular signaling cascade which helps to control the vital cellular functions of RTKs [203].

Aberrant RTKs activation such as receptor overexpression, chromosomal translocation, gene amplification, mutations and impaired receptor downregulation contribute to development of different forms of cancer in humans [204-206]. RTK deregulation and association to cancer formation have been reported in more than 30 RTKs [200]. Mutations and alternations in the structure of RTKs cause these receptors to become potent oncoproteins leading to neoplastic transformation [200, 207].

RTKs are viewed as multifunctional targets and strategies towards the inhibition of RTK signaling include monoclonal antibodies and small molecule inhibitors of tyrosine kinase activity [208]. Some of the important RTKs families implicated in cancer are VEGFR (vascular endothelial growth factor receptor), ErbB/EGFR (epidermal growth factor receptor), PDGFR (platelet-derived growth factor receptor), FGFR (fibroblast growth factor receptor) and IGFR (insulin growth factor receptor) families.

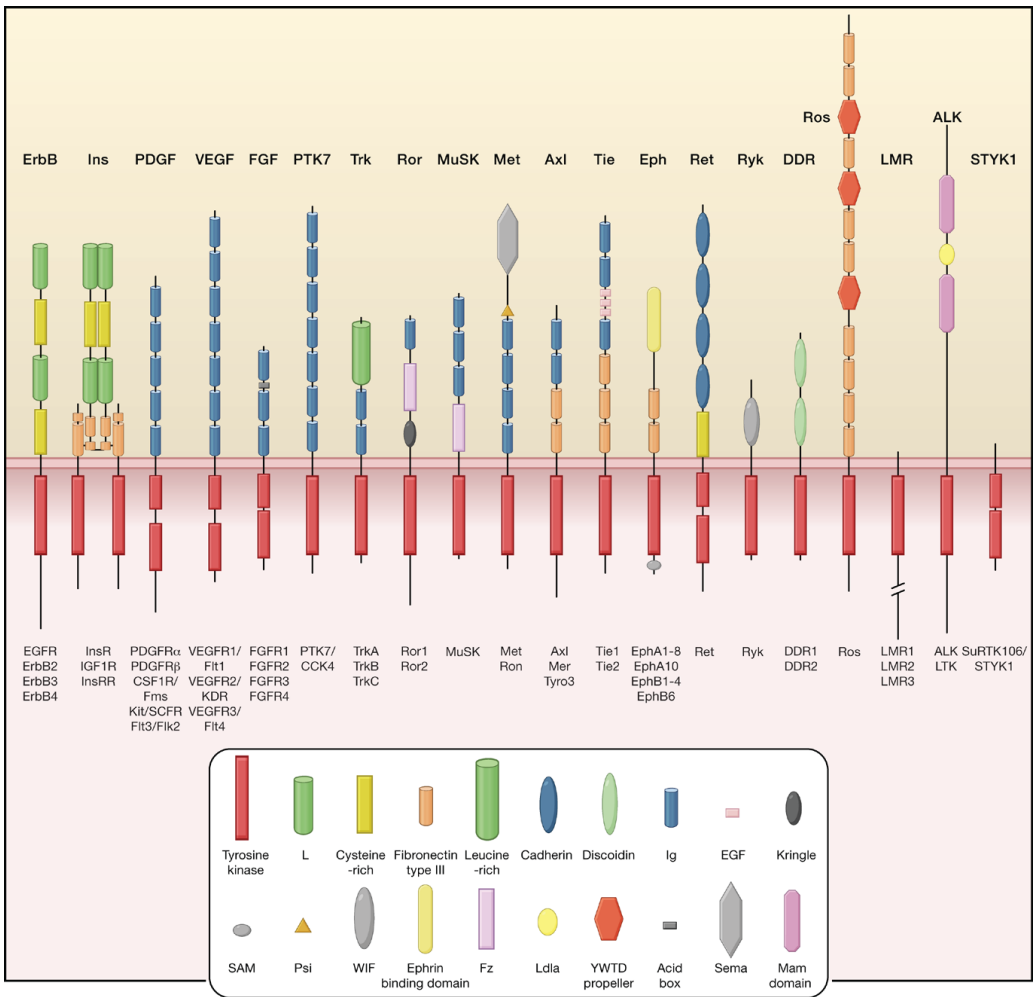

Figure 3. Receptor Tyrosine Kinase Families.

Reprinted from Cell, volume 141, issue 7. Lemmon MA et al, "Cell Signaling by Receptor Tyrosine Kinases" pp. 1117-1134. Copyright (2010), with permission from Elsevier.

2.2 STRUCTURE

Receptor tyrosine kinases structure consist of mainly three distinct regions, an extracellular region that binds polypeptide ligands, a transmembrane helix, and a conserved cytoplasmic region that possesses tyrosine kinase catalytic activity [209].

2.2.1 Extracellular region of RTKs

The extracellular region of RTKs is preceded by a cleavable signal sequence [210]. The extracellular part contains the binding sites for RTK ligands. These ligands range from soluble factors and extracellular matrix proteins to surface proteins expressed on adjacent cells. The extracellular domain may also be involved in the dimerization of RTKs, a process critical for the activation of intrinsic tyrosine kinase activity [211]. Most of the potential N-linked glycosylation sites are concentrated in the extracellular region [212,

213]. This domain is characterized by a low degree of sequence conservation. The extracellular region of RTKs contains a linear array of discrete globular domains such as immunoglobulin (Ig)-like domains, kringle (KNG) domains, fibronectin type III– like domains, cysteine-rich (CRD) domains, leucin-rich domains, cadherin-like domains, discoidin-like domains and EGF-like domains [209].

RTKs show structural differences in their extracellular regions with regard to the presence or absence of certain motifs. To give some examples, the EGFR extracellular domain contains two cysteine-rich regions, the PDGFR extracellular domain consists of five immunoglobulin-like repeats and the VEGFR extracellular domain contains seven consecutive immunoglobulin-like repeats. The extracellular domains of members of the insulin receptor subfamily contain disulfide bridges responsible for the formation of kringle domains and leucine-rich motifs [211].

2.2.2 Transmembrane region of RTKs

The transmembrane region of RTKs consists of a hydrophobic segment of 22–26 amino acids inserted in the cell membrane. This part of RTKs show a low degree of homology even between closely related RTKs. Lack of sequence conservation might suggest that specific amino acid residues within the transmembrane segment do not play a specific role in signal transfer. The transmembrane domain is flanked by a proline residue at their N-terminus and a cluster of basic amino acids in their C-terminus. Such a configuration is found in most transmembrane domains of RTKs and probably secures the transmembrane region within the lipid bilayer [211, 214, 215].

2.2.3 Cytoplasmic region of RTKs

The cytoplasmic portion of RTKs consists of a juxtamembrane region (just after the transmembrane helix), followed by the tyrosine kinase catalytic domain and a carboxy-terminal region. These regions contain tyrosine residues which are autophosphorylated upon ligand binding, regulate catalytic function and also serve as docking sites for SH2 (Src Homology 2) domain containing proteins.

2.2.3.1 Juxtamembrane domain

The juxtamembrane domain is a 41-50 amino acids segment that separates the transmembrane and cytoplasmic domains and is not well conserved between different families of receptors. However among members of the same family juxtamembrane domain shows a high degree of homology. This region may have a function in modulation of receptor functions by heterologous stimuli [208, 215, 216]. It probably also have a role in signaling suggested by capability to bind specific substrates in a ligand-dependent manner [208].

2.2.3.2 Tyrosine kinase domain

Of all the regions of RTKs, the tyrosine kinase domain shows the highest conservation level. This conserved tyrosine kinase part is not only within the same RTK family, but also when compared to other RTKs families [217]. Tyrosine kinase domain is evolutionary highly conserved among different species. To maintain a functioning receptor signaling, an intact tyrosine kinase domain is of high necessity. Even a single lysine mutation in the ATP-binding site can block the receptor ability to phosphorylate tyrosine residues thereby completely inactivating the biological function of the receptor. This region also contains autophosphorylation sites that are capable of coupling with signal-transducing molecules [208, 215, 218].

2.2.3.3 Carboxyl-terminal tail

The carboxyl-teminal tail is RTKs most distal and noncatalytic part. This region has the highest degree of heterogeneity in length (70-200 amino acids) and primary structure, even among members of the same RTK subclass. The carboxy-terminal tail is thought to contain tyrosine residues that are phosphorylated by the activated kinase part. Some reports have suggested an important role of C-terminal portion in modulating kinase activity [208, 215].

2.3 ACTIVATION AND REGULATION OF RTKs

Receptor tyrosine kinases are inactive, unphosphorylated and monomeric (with the exception of insulin growth factor receptor (IGFR) family of RTKs) in the absence of ligands and the conformation of the kinase domain is inactive [219]. The cytoplasmic juxtamembrane part has autoinhibitory effects on kinase activity of tyrosine kinase domain in some of the RTKs [220].

Upon ligand binding to extracellular region of RTKs, these receptors are activated and undergo a series of changes like receptor oligomerization, disruption of the autoinhibitory juxtamembrane interaction and autophosphorylation of specific tyrosine residues within the activation loop of the kinase domain [203]. Autophosphorylation creates binding sites for signaling proteins, recruiting them to the membrane and by doing so activate multiple signaling pathways [201].

2.3.1 Receptor dimerization

There is much evidence showing that activation of the kinase domain is ligand–induced and is mediated by receptor dimerization [221, 222]. Receptor tyrosine kinases dimerization happens either between two identical receptors (homodimerization), or between different members of the same receptor family (heterodimerization) [203]. Heterodimerization of RTKs is reported to increase the repertoire of ligands recognized by each receptor as well as expand signaling pathways diversity recruited by a given receptor [223, 224].

Until recently RTKs were thought to have a straightforward mechanism for ligand-induced dimerization, that is interaction of a bivalent ligand simultaneously with two receptor monomers and crosslinking them into a dimeric complex [204]. Some of the examples for this "ligand-mediated" mode of receptor dimerization are the nerve growth factor (NGF)/neurotrophin receptor TrkA, Tie2, Axl and Eph receptors [225-227].

Dimerization of RTKs is not restricted to the above mode only. There is "receptor mediated" dimerization where the ligand actually makes no direct contribution to the dimer interface. This is seen in the epidermal growth factor receptor (ErbB/EGFR) family of RTKs [228, 229]. The extracellular regions of ErbB receptors contain four domains (I–IV). The ligand binds to two distinct sites (on domains I and III) within a single receptor molecule. This ligand binding promotes conformational changes in the extracellular domain of ErbB receptors, unmasking a dimerization arm in domain II [230].

In the absence of a ligand, domain II is unavailable to both ligand binding and dimerization because it interacts with domain IV to stabilize a "tethered" conformation. This tethered conformation has an autoinhibitory effect on ligand binding and dimerization processes as it masks dimerization arm of domain II [231-233]. Ligand binding unfolds the tether, allowing domain II dimerization arm to interact with a second ligand-bound receptor molecule [204].

This is quite interesting in the sense that ErbB receptors are in fact different in their ligand binding capabilities. The ErbB/EGFR family consists of four closely related type I transmembrane tyrosine kinase receptors: EGFR/ErbB-1; also known as HER1, ErbB-2 (HER2), ErbB-3 (HER3) and ErbB-4 (HER4) [201]. There are known ligands for ErbB-1 and ErbB-4 and these receptors possess active tyrosine kinase domains. ErbB-3 lacks tyrosine kinase activity but it can bind to several ligands [224, 234, 235]. ErbB-2 (HER2) also has the active tyrosine kinase domain like ErbB-1 and ErbB-4 but is different from other members as there is no known ligand for it [230, 234]. In contrast to other members of ErbB RTK family, HER2 receptor exists in an active extended ('open') conformation and is permanently available for dimerization, as a result of this open conformation it does not need a ligand for activation (Figure 4) [236].

Dimerization can also involve a combination of ligand-mediated and receptor-mediated components, this is particularly seen in KIT and FGFR families [203, 237, 238]. Most RTKs dimerization is likely to be one of these three dimerization modes, ligand-mediated, receptor-mediated or a combination of ligand-mediated and receptor-mediated components [204].

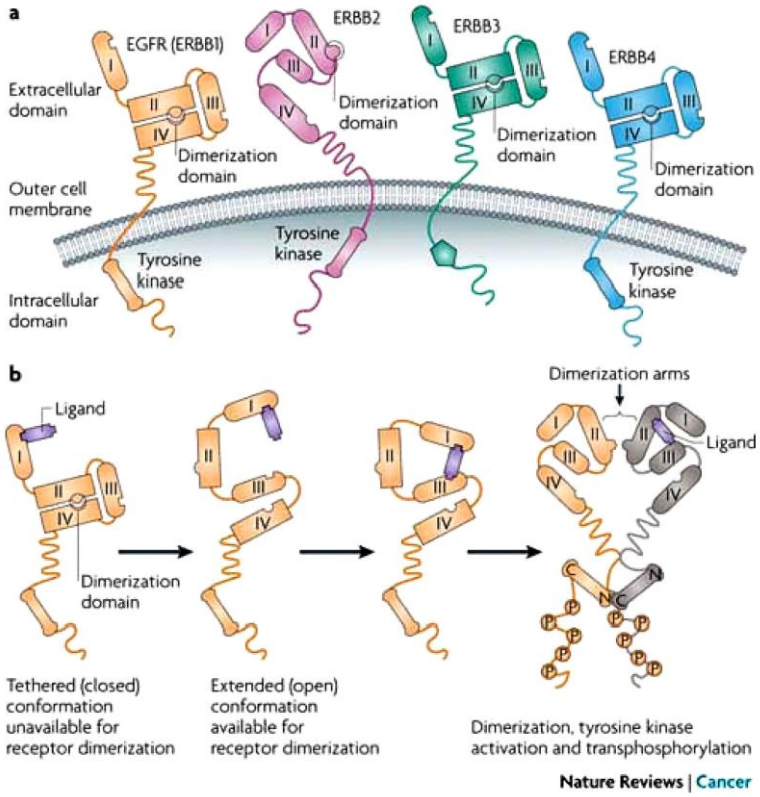

Figure 4. ErbB receptors. a) Four members of the ErbB family, EGFR, ERBB3 and ERBB4 exist in a tethered ('closed') conformation in which the dimerization domain is not available to interact with partner ERBB moieties in the absence of ligand. ERBB2 exists in an active extended ('open') conformation and is permanently available for dimerization. b) Conceptualization of the receptor conformational change on ligand binding.
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2.3.2 Tyrosine kinase autophosphorylation

Autophosphorylation of RTKs occur in two ways: *cis*-phosphorylation (within a receptor) or *trans* (between receptors) phosphorylation. In the first instance, dimerization initiated by ligand-binding causes a conformational change in the receptor, facilitating *cis*-autophosphorylation. In the second case, there is no conformational change upon ligand-binding, just a simple proximity effect provides sufficient opportunity for trans-autophosphorylation to occur [209].

Receptor tyrosine kinases require two processes for their intracytoplasmic activation: a) increase in intrinsic catalytic activity and b) creation of docking sites for recruiting downstream signaling proteins. These two processes are accomplished for the majority of RTKs by autophosphorylation on tyrosine residues, a consequence of ligand-mediated oligomerization.

The activation loop within the kinase domain is where autophosphorylation of tyrosine residues takes place and this result in stimulation of kinase activity and autophosphorylation of tyrosines in the juxtamembrane and carboxy-terminal regions. All these phosphorylation steps lead to generation of binding sites for modular domains that

specifically detect phosphotyrosine in specific sequence contexts. Src Homology 2 (SH2) and the phosphotyrosine-binding (PTB) domains are two well-known phosphotyrosine binding modules present within signaling proteins [239]. The activation loop in the kinase domain contains between one to three tyrosine residues in many RTKs [240].

There are reports on a number of RTKs, showing that the phosphorylation of these tyrosine residues in the activation loop is essential for stimulation of catalytic activity and biological function of RTKs. Some of the RTKs families studied in this regard include: insulin growth factor receptor (IGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), hepatocyte growth factor receptor (Met) and TrkA [241-246]. Only epidermal growth factor receptor (EGFR) is the major exception to catalytic enhancement via activation loop autophosphorylation [247].

2.3.3 Receptor tyrosine kinases regulation

As receptor tyrosine kinases play critical roles in cellular signaling processes, there is a need for their catalytic activity to be under control. The first level of regulation is created by the kinase domain itself where the state of phosphorylation directly controls kinase activity [248]. Different regions of intracellular domain of RTKs exhibit autoinhibitory mechanisms which then help in keeping the catalytic activity of RTKs under firm control. A second level of RTKs regulation is achieved by their downregulation. Receptor tyrosine kinases after activation by their ligands and conducting their signal transduction, are rapidly internalized and degraded helping to quickly terminate the action of the ligands. These mechanisms are discussed below.

2.3.3.1 Autoinhibition by activation loop of tyrosine kinase domain

One of these autoinhibitory effects comes from the activation loop within the tyrosine kinase domain itself. There exists a tight control of catalytic tyrosine kinase activity resulting from a mechanism known as *cis*-inhibition/*trans*-activation [209]. In this concept, each tyrosine kinase domain (activation loop) is exclusively *cis*-autoinhibited by a series of intramolecular interactions specific for its receptor. Ligand binding and receptor dimerization promotes *trans*-phosphorylation of activation loop tyrosine residues, which then disrupts the *cis*-autoinhibitory interactions and promotes receptor activation.

An equilibrium is always present between inactive and active loop conformations of the unphosphorylated RTKs [248]. This balance allows *cis*-inhibition, but at the same time permits *trans*-phosphorylation between ligand induced receptor dimers [200]. The key event triggering RTK activation seems to be the release of *cis*-autoinhibition, following ligand-induced receptor dimerization. Studies have revealed that insulin growth factor receptor (IGFR) was the first receptor to demonstrate RTK autoinhibition in the activation loop of their tyrosine kinase domain [249]. Fibroblast growth factor receptor-1 (FGFR1) is another example of this *cis*-inhibition/*trans*-activation mechanism [250, 251].

2.3.3.2 Autoinhibition by juxtamembrane domain

Juxtamembrane autoinhibition is a phenomenon where RTKs are *cis*-autoinhibited by components outside their tyrosine kinase domain. In this case, key tyrosines in the juxtamembrane region make extensive contacts with different parts of the tyrosine kinase domain, including the activation loop and help to stabilize an autoinhibited conformation. This event is seen in MuSK [252], Flt3 [220], KIT [253] and Eph family RTKs [254].

2.3.3.3 Autoinhibition by C-terminal domain

This form of autoinhibition is seen in Tie2 RTK and possibly in Met and Ron RTKs. In Tie2, an inactive conformation is maintained by interaction of a region in the C-terminal domain containing tyrosine autophosphorylartion sites with the active site of the tyrosine kinase domain, blocking the access of protein substrates to the activate site and thus stabilizing the inactive conformation [255].

2.3.3.4 Downregulation of receptor tyrosine kinases

In addition to the regulatory mechanisms discussed above for controlling the tyrosine kinase activity of RTKs, another important means of RTKs regulation in cells is their downregulation. This process involves ligand-stimulated endocytosis of occupied RTKs and ensuing intracytoplasmic degradation of both the ligand and receptor molecules and have been studied extensively for ErbB receptor tyrosine kinases [204, 256-259]. Endocytosis can happen either through clathrin dependent or clathrin independent mechanisms.

Recent studies have revealed that ubiquitylation of proteins is regulated by the phosphorylation process and in turn ubiquitylation regulates the function of a variety of protein kinases and phosphatases [260, 261]. Furthermore, the crosstalk between these two important posttranslational modifications (PTMs) plays a vital role in regulating protein degradation, processing and cellular trafficking [204, 262].

2.4 RECEPTOR TYROSINE KINASES AND CANCER

Oncogenic deregulation seems to be the result of disruption of one or several of the auto control mechanisms that secure the normal repression of catalytic domains. More than thirty of known RTKs are implicated in different human malignancies. Aberrant tyrosine kinase activation increases the survival, proliferation rate and drug resistance of malignant cells. It is also responsible for the increase in angiogenesis, invasiveness and metastatic potentials of tumors [200, 219].

As discussed earlier regulation of tyrosine kinases occurs in multiple levels, so it is not surprising that their dysregulation in cancer cells also happens in several ways. The main principles for oncogenic transformation of RTKs are a) gain-of-function (GOF) mutations or small deletions, b) overexpression resulting from gene amplification and c) genomic re-arrangements, such as chromosomal translocations. The first important mechanism of aberrant tyrosine kinase activation is mutations (gain-of-function) or small deletions that

disturb autoregulation of the kinase domain [219]. The examples of mutations in RTKs and their link to cancer can be found in many malignancies. One such example is the mutation of FLT3 receptors in acute myeloid leukemia (AML). These mutations results in activation of tyrosine kinase domain in the absence of the ligand [263]. In another example, point mutations and small deletions in the kinase domain of epidermal growth factor receptor (EGFR) in non–small-cell lung cancers (NSCLC) increase the sensitivity of the receptor to its ligand and causes alternation in receptor signaling [264-266]

The second mechanism of deregulation of tyrosine kinase is the overexpression of RTKs due to possible gene amplifications. Receptor tyrosine kinase overexpression leads to constitutive kinase activation by increasing the concentration of dimers. This also results in enhanced expression of these receptors as well as availability of specific ligands. Examples include overexpression of ERBB2 (HER2/neu) RTK in breast cancer [267, 268], overexpression of ROR1 receptor tyrosine kinase in chronic lymphocytic leukemia (CLL) [269-271].

The third mechanism which is also a common mechanism of oncogenic deregulation in hematologic malignancies is the fusion of a receptor or non-receptor tyrosine kinase with a partner protein, usually the result of genomic re-arrangements, most often a balanced chromosomal translocation. The partner protein has a distinguished feature in a form of a domain that causes constitutive oligomerization of the RTK in the absence of the ligand binding, thereby contributing to autophosphorylation and activation [219]. A well-known example of this mechanism is a reciprocal translocation between chromosomes 9 and 22 that generates the Philadelphia (Ph) chromosome. At the molecular level, this translocation juxtaposes the coding sequences of the bcr and c-abl genes (c-abl gene encodes a non-receptor tyrosine kinase). This genetic fusion creates an oncoprotein called BCR-ABL, with constitutively active tyrosine kinase activity [272].

2.5 TARGETING RECEPTOR TYROSINE KINASES IN CANCER

There are two major ways of targeting RTKs active in malignancies of both hematological and solid tumors. Monoclonal antibody therapy and small inhibiting molecules have been two successful approaches in cancer treatment strategies.

2.5.1 Therapeutic antibodies

One of the important mechanisms in targeting RTKs in cancer is by using antibodies against aberrant receptor tyrosine kinases or their ligands. Antibody targeting results in disruption of tyrosine kinase signaling through neutralization of ligand, obstruction of ligand binding, receptor internalization and degradation and perhaps antibody-mediated cytotoxicity.

Monoclonal antibodies have been developed against different RTKs and their ligands in a variety of cancers. Antibodies against RTKs function mainly either as neutralizing antibodies binding to activating ligands of RTKs or anti-dimerization antibodies preventing homo- and hetero-dimerization of RTKs (Figure 5).

The ErbB-2 or HER2 receptor tyrosine kinase is overexpressed in about 30% of invasive breast cancers through gene amplification and is associated with a poor prognosis. We know that HER2 has no known ligand and trastuzumab which targets HER2 exert its function by inhibiting receptor homo- and hetero-dimerization, internalization and endocytic destruction, rather than by blocking ligand binding [273, 274]. Trastuzumab is a humanized IgG1 mAb and is used for the treatment of metastatic HER2-overexpressing breast cancer and shows a 35% response rate in patients with metastatic breast cancer who did not receive prior chemotherapy [275-277]. Trastuzumab in combination with cisplatin and a fluoropyrimidine is recommended for advanced gastric cancer positive for the human epidermal growth factor receptor 2 (HER2/neu) [278].

Cetuximab is a chimeric EGFR-specific IgG1 mAb used as second- or third-line therapy for the treatment of metastatic colorectal cancer [279] and squamous cell carcinoma of the head and neck [280]. Cetuximab functions by inhibiting the binding of activating ligand and also by prevention of receptor dimerization leading to disruption of the signal transduction cascade [277, 281, 282].

Panitumumab is a fully humanized IgG2 mAb that is specific for EGFR and like cetuximab is used as second- or third-line therapy for the treatment of metastatic colorectal cancer [279]. It works by a mechanism similar to cetuximab but in contrast to cetuximab it does not promote ADCC [277]. The vascular endothelial growth factors (VEGFs) are expressed by many solid tumors and bind to its receptors on the vascular endothelium resulting in angiogenesis. Benefit from cetuximab and panitumumab is restricted to patients with wild-type K-ras tumors. One of the most important selection criteria for cetuximab and panitumumab therapies is the status of K-ras [283].

Bevacizumab is a humanized mAb specific for VEGF and it prevents binding of VEGF to its receptor and is approved for the treatment of breast [284], colorectal [285] and non-small-cell lung [286] carcinomas in combination with cytotoxic chemotherapy [277, 287]. Bevacizumab is also used in combination with IFN in the treatment of patients with metastatic renal cell carcinoma (mRCC) [288]. There are other monoclonal antibodies in various stages of clinical testing but not yet approved by the authorities.

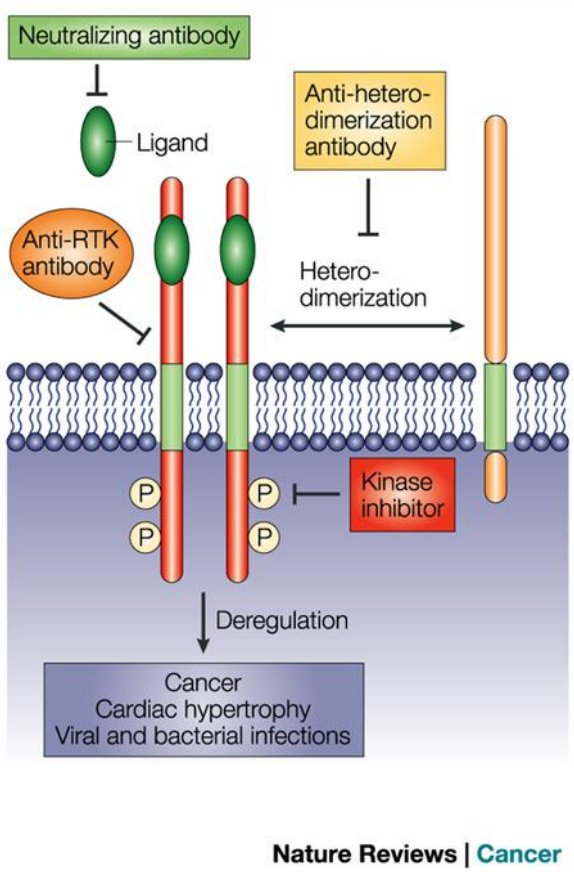

Figure 5. Receptor tyrosine kinases: sites of therapeutic intervention. Neutralizing antibodies, which block the bioactivity of RTK ligands, RTK-targeted antibodies, which either target overexpressed receptors or receptor heterodimerization, and small-molecule inhibitors of RTK kinase activity have been developed to interfere with RTK signal transduction.

2.5.2 Small-molecule inhibitors

Small-molecules inhibitors can translocate through plasma membrane and by interacting with the cytoplasmic domain of RTKs inhibit the catalytic activity of the tyrosine kinase domain by interfering with the binding of ATP or substrates. Some of the important and approved small-molecule agents are briefly mentioned in this section.

Imatinib is one of the first successful small-molecule inhibitors, it inactivates the kinase activity of the BCR–ABL fusion protein in chronic myelogenous leukemia (CML) [289, 290]. In addition to BCR–ABL, platelet-derived growth factor receptor (PDGFR) receptor tyrosine kinase is also found to be effectively inhibited by imatinib [291]. Imatinib acts as a multi-targeted small-molecule inhibitor of other RTKs including KIT which was found to have important roles in the pathogenesis of gastrointestinal stromal tumours (GISTs) [292].

Gefitinib [293] and erlotinib [294] are potent and selective inhibitors of EGFR tyrosine kinase activity. Both these small-molecule agents are approved for treatment of EGFR expressing cancer non-small-cell lung cancer (NSCLC) [295, 296]. Erlotinib is also approved for treatment of advanced pancreatic cancer in combination with gemcitabine [297].

3 ROR FAMILY OF RECEPTOR TYROSINE KINASES

3.1 INTRODUCTION

Receptor tyrosine kinases (RTKs) are important mediators in different cellular processes such as proliferation, differentiation, angiogenesis, survival and migration. Interaction of a ligand with extracellular domains of RTKs facilitate communication between the cell and its environment and lead to initiation of an intracellular signaling cascade.

The ROR family of RTKs are type I transmembrane proteins and are largely located in the plasma membrane [298]. The vertebrate ROR RTKs consist of two structurally related family members, ROR1 and ROR2 (formerly known as neurotrophic tyrosine kinase receptor (NTRKR1) and (NTRKR2), respectively) [299]. These receptors are evolutionarily conserved in invertebrate and vertebrate, including fruit flies, roundworms, sea slugs, zebrafish, chickens, frogs, mice, rats and humans.

The ROR protein extracellular region contains an immunoglobulin (Ig) domain, a Frizzled-like cysteine-rich domain (CRD) and a membrane-proximal kringle (KNG) domain. Intracellularly, ROR proteins possess a tyrosine kinase (TK) domain and a proline-rich domain (PRD) straddled by two serine/threonine-rich domains, serine/threonine-rich domain 1 and serine/threonine-rich domain 2 (Figure 6) [300]. The characteristic features of ROR family of RTKs are the presence of intracellular tyrosine kinase (TK) domains, related to those of the Trk-family RTKs, extracellular Frizzled-like cysteine-rich domains (CRDs) and membrane-proximal Kringle domains [301-304].

Many members of ROR family were isolated by polymerase chain reaction (PCR) based screens for tyrosine kinase encoding genes. The first ROR family members, two human ROR RTK-encoding genes (*hRor1 and hRor2*) were originally identified by polymerase chain reaction (PCR) based screens nearly 20 years ago in a human neuroblastoma cell line [300].

For quite some time, the receptor tyrosine-like orphan receptor (ROR) family of RTKs was among a minority of RTKs families, for which the ligand and signaling pathway was unknown, giving rise to their orphan nomenclature. Recent studies have revealed a lot of information about functions of ROR family of RTKs and in particular have shown that ROR proteins are in fact Wnt receptors. We know that dysfunctions of RTKs results in severe developmental defects and diseases such as cancer. Human ROR proteins mutations and dysregulations are associated with skeletal deformities and leukemia [305].

The presence of a truncated ROR1 molecule has been reported in human neural tissues, which lacks the extracellular and transmembrane parts. This isoform truncated ROR1 (t-ROR1) was noted in fetal, adult human CNS, leukemia/lymphoma cell lines and in a variety of human cell lines derived from neuroectoderm [11]. Most species appear to contain two ROR genes. For example, Drosophila, mouse, rat and human genomes all contain two ROR genes. In contrast, only a single ROR-encoding gene has been found in Caenorhabditis elegans [306].

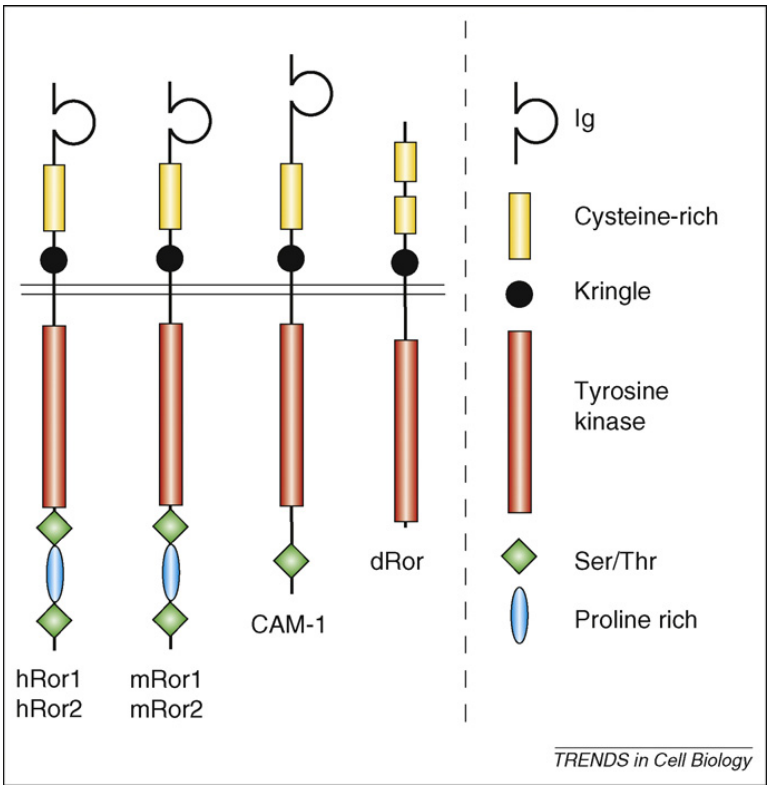

Figure 6. Structure of ROR receptor tyrosine kinases (RTKs) in different species. Domain organization of ROR proteins in human (hROR1, hROR2), mouse (mRor1, mRor2), C. elegans (CAM-1) and Drosophila (dROR). The N-terminal extracellular domain (ECD) is above and the intracellular domain (ICD) is below the double line representing the plasma membrane.

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ROR orthologs have also been identified in fruit flies (*Drosophila melanogaster; dROR*) [307], roundworms (*Caenorhabditis elegans; cam-1*) [308, 309], sea slugs (*Aplysia californica; Apror*) [310], zebrafish (*Danio rerio; Ror2 and Ror2*) [311], chickens (*Gallus gallus; cRor1 and cRor2*) [312, 313], frogs (*Xenopus laevis; XRor1 and XRor2*) [314], mice (*Mus musculus*; *mRor1 and mRor2*) [301] and rat (*Rattus norvegicus*; *rRor1 and rRor2*) [11]. Whereas the CRD, kringle and TK domains are characteristic of all ROR proteins, the architecture of the other domains varies between species [305].

Here in this section of the thesis, I will concentrate mainly on ROR RTKs of mammals, with special attention to human ROR1 and ROR2 receptor tyrosine kinases structure, function, expression pattern and involvement in human diseases.

3.2 STRUCTURE OF ROR1 AND ROR2 RECEPTOR TYROSINE KINASES

ROR1 and ROR2 from each species are homologous to one another. Human ROR1 and ROR2 *(hRor1* and *hRor2)* share an overall 58% identity in their amino acid sequence. This amino acid identity is reported to be 68% within their kinase domains. The degree of sequence conservation is higher within the ROR1 and ROR2 subgroups. For example, a 97% amino acid identity overall is shared between human and mouse ROR1 *(hRor1* and *mRor1).* For ROR2 *(hRor2* and *mRor2)* the identity overall is 92% [11, 306]. Murine

RORs are the same length as human, except that murine ROR2 (*mRor2)* is one amino acid longer than human ROR2 (*hRor2)*.

Mammalian ROR proteins have the same domain structure between them; they contain extracellular immunoglobulin (Ig), cysteine-rich (CRD) and kringle (KNG) domains, and intracellularly they share tyrosine kinase (TK), proline rich and two serine-threonine rich domains in addition to their primary sequence homology and similarity in size. ROR receptors are evolutionarily highly conserved between different species e.g., human, mouse, Drosophila and C. elegans, suggesting important biological functions.

Human ROR1 is located on chromosomal region 1p31.3 and consists of 937 amino acids (908 after cleavage of the signal peptide) with the estimated molecular weight of 104.28 kDa. Human ROR2 is situated on chromosomal region 9q22 and consists of 943 amino acids (910 after signal peptide cleavage) with the molecular weight estimated to be 104.76 kDa.

As discussed above ROR family of RTKs consist of three regions, an extracellular region, a transmembrane region and an intracellular region. The extracellular region contain immunoglobulin (Ig), cysteine-rich (CRD) and kringle (KNG) domains, motifs that are believed to participate and mediate protein-protein interactions. The intracellular region of RORs contain a tyrosine kinase domain, two regions rich in serine and threonine separated by a region rich in prolines [306].

3.2.1 Extracellular regions of ROR RTKs

3.2.1.1 Signal peptide of ROR RTKs

ROR family members contain a predicted short signal sequence of approximately 20 amino acids at their N-termini. The signal peptides of human ROR1 and ROR2 consist of 29 and 33 amino acids respectively. Signal peptide is quite essential because it directs the protein where it should go. If a given protein is for secretion, the signal peptide directs the protein to the endoplasmic reticulum where it will grow further and mature for secretion. The signal peptide is recognized by the signal recognition particle (SRP) and cleaved by the signal peptidase following transport at the endoplasmic reticulum [306, 315].

3.2.1.2 Immunoglobulin-like domain (Ig)

Immunoglobulin-like (Ig) domain of human ROR1 consist of 106 amino acids residues, the corresponding number for ROR2 is 91 amino acids. The precise function of the Immunoglobulin-like domain is not known but it might be involved in protein–protein and/or protein–ligand interactions. The other possibility for the role of Ig domain might be modification of the function of CRD and Kringle domains [316].

3.2.1.3 Cysteine-rich domain (CRD)

ROR proteins extracellular cysteine-rich domain (CRD) is defined by the presence of 10 conserved cysteines that form five disulfide bridges. Human ROR1 and ROR2 contain a

cysteine-rich domain of 135 amino acids each. The cysteine-rich domain (CRD) of ROR RTKs is similar to the Wnt binding domain (CRD region) of Frizzled receptors and is considered one of the ligand binding motifs of ROR receptor tyrosine kinases, the other is thought to be the kringle domain.

There are studies now indicating that ROR RTKs also bind to Wnt proteins (ligands) and act as their receptors [299, 305]. ROR1 and Wnt5a have been shown to physically interact with each other and cooperatively activate NF-κB when overexpressed in HEK293 cells. Survival of CLL cells in vitro is enhanced by Wnt5a, an effect that could be neutralized by anti-ROR1 antisera. These findings indicate to the fact that ROR1 functions as an oncofetal surface antigen through which Wnt5a activates NF-κB–dependent survival signaling in CLL [271]. It is also reported that ROR2 acts as a receptor or co-receptor for Wnt5a and the CRD of ROR2 is required for binding to Wnt5a and mediating Wnt5a signaling to cell interior [317, 318].

3.2.1.4 Kringle domain (KNG)

The membrane proximal kringle domains of ROR1 and ROR2 consist of 80 and 79 amino acids sequence respectively. It is also highly conserved throughout the ROR family RTKs and might function as recognition modules for binding to other proteins including Wnt regulatory proteins and possibly ROR ligands [299]. The presence of a kringle domain is a nearly diagnostic characteristic of ROR family members as ROR is the only RTK family reported to contain the kringle domain with the exception of Torpedo MuSK [306].

3.2.2 Intracellular regions of ROR RTKs

3.2.2.1 Tyrosine kinase domain (TKD)

ROR receptor tyrosine kinases contain 40 amino acids within the kinase domain [240, 319]. Twenty one amino acid out of the forty consensus amino acids, are absolutely conserved among all RTKs studied until now, making this region the highest conserved region across different species of not only ROR receptor tyrosine kinases family but also in all known RTKs [240].

Vertebrate RORs show the most prominent changes in the conserved amino acids. Seven amino acids in human ROR1 and mouse ROR1 are altered, the corresponding number of amino acid alternations for human ROR2 is five and for mouse ROR2 is six. Only a single conserved amino acid is altered in each of the invertebrate RORs, in contrast to the vertebrate RORs [306]. It was thought that due to numerous changes to highly conserved amino acids within mammalian ROR kinase domains, the kinase activity of ROR RTKs may have been lost. This has been investigated and despite their lack of several amino acids that are highly conserved in RTKs, ROR1 and ROR2 each have kinase activity in vitro [300, 301].

3.2.2.2 Serine/threonine-rich (S/TRD) and prolin-rich domains (PRD)

ROR1 and ROR2 both possess two serine/threonine-rich domains (S/ TRD1) and (S/ TRD2) on both sides of a proline-rich domain (PRD) at the C-terminal to the TK domains. No similar domains to the S/TRDs or PRDs of the ROR family RTKs have been found in any other proteins. ROR1 and ROR2 serine/threonine-rich domains 1 (S/TRD1s) show a high degree of homology (~67% identity) but the S/TRD2s do not exhibit any apparent homology. The proline-rich domains (PRDs) demonstrate a lower degree of homology (~30% identity). These cytoplasmic domains are believed to take part in the signaling mechanism of the ROR family RTKs by interacting with signaling mediators [299]. Within these serine/threonine-rich and proline-rich domains, it is thought that there are potential phosphorylation sites and Src homology 2 (SH2) and Src homology 3 (SH3) recognition motifs for protein interaction. These motifs might be important in ROR mediated signal transduction by regulating association with SH2 and SH3 domains of adaptors/signaling molecules [320].

3.3 ROR EXPRESSION AND FUNCTION

The human ROR1 is expressed in fetal heart, lung and kidney and to a lesser extent in placenta, pancreas and skeletal muscles [11]. Normal adult tissues including brain, breast, testis, ovary, uterus, prostate, lymph node, small intestine, heart, liver, lung, kidney, pancreas, adipose tissue, skin, spleen, adrenal gland, amygdala, pituitary gland, placenta, bladder, tonsil, stomach, colon, rectum, skeletal muscle, spinal cord and eye were investigated for ROR1 protein expression using a commercial polyclonal goat anti-human ROR1. No ROR1 expression (expected band of ROR1 ~120 kDa) was detected in majority of these twenty eight normal adult tissues except for a very low level of ROR1 expression in testis, uterus, lung, bladder, and colon [269].

Moreover, in another study the relative expression of ROR1 mRNA was detected by real-time quantitative PCR in a number of normal hematopoietic and non-hematopoietic tissues including CD8+ T-cells, CD4+ T-cells, lymph node, tonsil, bone marrow, fetal liver, heart, brain, lung, liver, kidney, pancreas, spleen, thymus, prostate, testis, ovary, placenta, small intestine, colon, skeletal muscle, fibroblast and adipose tissue. ROR1 gene was not expressed at significant levels in normal adult non-hematopoietic tissues apart from low levels in pancreas and adipose tissue. Among normal adult hematopoietic it was transiently expressed at an intermediate stage of normal B-cell development at a level that is similar to that in B-CLL [321]. The human ROR2 is expressed in fetal heart, kidney and lung. In adults ROR2 is expressed in the uterus, prostate, ovary, thyroid gland and small intestine [316].

There are studies in mice showing that ROR1 and ROR2 are broadly expressed during embryonic developmental stages including skeletal, respiratory and cardiac developments as well as neurite extension in central neurons and their expression is tightly downregulated after birth [298, 300, 301, 322-324]. To study the functions of mouse ROR1 and ROR2 receptor tyrosine kinases, mice lacking either of these genes have been generated. ROR1 mutant newborn mice were similar in size to the wild-type mice and did not exhibit any

apparent gross abnormalities, yet after birth these ROR1 mutant mice showed respiratory dysfunction and cyanosis and died within 24 hour [316, 323].

In contrast, ROR2 mutant mice exhibited dwarfism, short limbs and tail and facial anomalies. These ROR2 mutant newborns also showed forced respiration and cyanosis and died within 6 hour after birth [325, 326]. ROR1 and ROR2 newborn mice died shortly after birth because of difficulty in breathing and the reason for this was found to be the incomplete expansion of the alveoli in these mice [316, 323, 326]. As ROR1 and ROR2 genes showed similar expression patterns in the developing face, limbs, heart and lungs [298], the absence of seeming morphological abnormalities in ROR1 mutant mice could be explained by functional redundancy between ROR1 and ROR2.

ROR1/ROR2 double mutant mice were generated to find out if ROR1 interacts genetically with ROR2 during developmental morphogenesis. Most of ROR1/ROR2 mutant mice died before delivery showing the process of perinatal lethality. Despite the fact that overall appearance of ROR1 mutant mice appeared to be identical to that of wild-type mice, ROR1/ROR2 mutant mice exhibited greater ROR2 mutant phenotypes. The limb and tail shortening in proportion to the body length and facial anomalies witnessed in ROR2 mutant mice were more profound in ROR1/ROR2 mutant mice, indicating that ROR1 and ROR2 could interact genetically during developmental morphogenesis [316, 323].

3.4 ABERRANT ACTIVATION OF ROR RECEPTOR TYROSINE KINASES

The principal mechanisms leading to aberrant RTK activation in human cancers are, autocrine activation, chromosomal translocations, RTK overexpression and gain-of-function mutations. ROR1 aberration in different cancers, both hematological malignancies and solid tumors, has been due to the overexpression of this RTK. There are so far no studies showing any mutations or chromosomal translocations of ROR1 receptor tyrosine kinase in any cancer. In contrast ROR2 seems to have mutations as well as overexpression as the mechanisms for its aberrant activity. ROR2 mutations have been reported in humans and cause skeletal defects anomalies. Several solid tumors also show overexpression of ROR2 RTK.

3.4.1 ROR1 overexpression and cancer

A gene expression profiling study of chronic lymphocytic leukemia (CLL) in 2001 revealed down and upregulation of a number of genes in CLL. Among the genes upregulated in CLL, there was ROR1 receptor tyrosine kinase with a 43.8-fold increase in CLL cells [327]. Since publishing the results of gene profiling study, special attention has been directed towards investigating the role of ROR1 in CLL. One important reason for the interest in studying the relationship between ROR1 and CLL was the success stories of other RTKs involved in other type of cancers and the possibility of targeted therapy in these cancers.

There were almost three simultaneous studies showing overexpression of ROR1 at the gene and protein levels in CLL cells of all patients. Healthy donor B cells, other blood cells of healthy donor and normal adult tissues did not express ROR1 [269-271]. The antibody binding capacity (ABC) of ROR1 on the surface of B-CLL cells was measured to be in the range of 10^3 to 10^4 molecules per cell (2,773-7,090 molecules per cell). There was no detectable soluble ROR1 protein in >75% of sera from B-CLL patients [269].

Mutation analysis of cloned extracellular and cytoplasmic kinase domains of the ROR1 gene showed no major genomic aberrations (mutation or truncation). No difference was detected in protein expression of ROR1 comparing IGHV mutated and unmutated cases. FISH analysis of CLL patients showed no rearrangement in the ROR1 locus [270]. The extracellular domain of ROR1 could bind Wnt5a molecule and coexpression of ROR1 and Wnt5a in HEK cells lead to the activation of NF-κB. Additionally, CLL cells viability cocultured with Wnt5a-expressing Chinese Hamster Ovary (CHO) cells was significantly greater than those cultured with CHO cells alone. This survival advantage was neutralized by the addition of antisera against ROR1 [271].

Acute lymphocytic leukemia (ALL) patients showed overexpression of ROR1 at gene level. A similar expression pattern was also observed in ALL cell lines [328, 329]. There are reports showing expression of ROR1 at gene and protein levels in mantle cell lymphoma and multiple myeloma cell lines [321, 330], in leukemic cells of marginal zone lymphoma (MZL), mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) patients [331]. Additionally, ROR1 was detected at protein level in patients with different B-cell non-Hodgkin lymphomas (B-NHL) including immunocytoma (IC), mantle cell lymphoma, marginal zone lymphoma and follicular lymphoma [332].

Recently, ROR1 has also been shown to be overexpressed in several solid tumors [333, 334]. The presence of a truncated ROR1 isoform (t-ROR1) lacking both the extracellular and transmembrane parts has also been reported in some leukemia/lymphoma cell lines in addition to human neural tissues, fetal and adult human CNS and in a variety of human cell lines derived from neuroectoderm [11].

3.4.2 ROR2 genetic disorders and cancer

Mutations in the human ROR2 gene cause two well characterized skeletal defects, recessive Robinow syndrome (RRS) [335, 336] and dominant brachydactyly B (BDB) [337, 338]. Recessive Robinow syndrome (RRS) is characterized by short-limbed dwarfism and brachydactyly and is caused by loss-of-function mutation of ROR2. Additionally these patients exhibit abnormal morphogenesis of face and external genitalia, segmental defects of the spine and rib fusions [339-342]. ROR2 mutations in recessive Robinow syndrome are in fact missense, nonsense, and frameshift mutations in CRD, kringle and kinase domains of ROR2 RTK, suggesting reduced or eliminated functions. The second skeletal disorder caused by mutations in ROR2 gene is brachydactyly B (BDB), inherited as an autosomal dominant disorder. Affected patients show hypoplasia/aplasia of distal phalanges and nails [343, 344]. Brachydactyly type B is

thought to be caused by specific mutations in ROR2 gene resulting in truncation of ROR2 either before or after the kinase domain.

ROR2, has been shown to be overexpressed in squamous cell carcinoma [345], malignant melanoma [346], osteosarcoma [347], renal cell carcinoma [348], prostate and gastric cancers [349, 350]. ROR2 was not detected in CLL and white blood cells of normal donors [269].

3.5 TARGETED THERAPIES OF ROR RECEPTOR TYROSINE KINASES

RTKs are considered as multitarget proteins and strategies towards the inhibition of RTKs signaling include monoclonal antibodies and small molecule inhibitors of tyrosine kinase activity. A number of therapeutic mAbs used in oncology are summarized in Table 6. Several studies have revealed the presence of two other RTKs expressed in CLL, VEGFR and Axl, in addition to ROR1. Additionally studies have indicated that CLL cells are capable of secreting VEGF spontaenously, mainly the isoforms $VEGF_{165}$ and $VEGF_{121}$ [351, 352] and constitutively express activated VEGF-R1 and VEGF-R2 .These observations might indicate that the apoptosis defect of CLL cells is partly mediated through an autocrine feedback loop involving VEGF [352]. Moreover, most of studies showed a positive correlation between the level of VEGF receptors on the cell surface and shorter survival in CLL patients [353]. Recently another RTK, Axl has been described to be constitutively phosphorylated in most CLL B cells [354].

Targeted therapies in the form of mainly small inhibiting molecules and to a lesser extent mAbs have been described for VEGF receptors in CLL. There are reports demonstrating utilization of four tyrosine kinase inhibitors (TKIs) in targeting VEGFRs [355-358]. Vatalanib and Pazopanib, two of these TKIs are specific inhibitors of VEGF receptors. Furthermore, these two TKIs could induce apoptosis of CLL cells both in vitro and in vivo (mice grafted with the CLL-like cell line JVM-3). Additionally there was a decrease in phosphorylation and downregulation of the antiapoptotic proteins XIAP and Mcl-1 [357].

In a phase II clinical trial three distinct anti-VEGF therapies were tested for patients with relapsed/refractory CLL. Between 2005 and 2008, 46 patients were recruited to trials of single-agent anti-VEGF antibody (bevacizumab, n=13) or one of two RTK inhibitors (AZD2171, n=15; sunitinib malate, n=18). A median of two cycles of bevacizumab, AZD2171, or sunitinib malate were given to patients and all patients completed the treatment course. However, no complete or partial response was detected and due to lack of efficacy all three trial were closed prematurely. In conclusion these studies demonstrated that single-agent anti-VEGF therapy has minimal effect for patients with relapsed/refractory CLL [356].

Furthermore, in another study sorafenib, a multikinase inhibitor induced apoptosis in primary CLL cells. Sorafenib-induced apoptosis led to downregulation of Mcl-1 and activation of caspase-3 and -9, indicating involvement of the cell intrinsic apoptotic pathway. Additionally bevacizumab failed to induce apoptosis in CLL cells, suggesting that

sorafenib might induce apoptosis irrespective of VEGF signaling [358]. A green tea component, epigallocatechin-3-gallate (EGCG) is a known TKI. The effect of this TKI on primary CLL cells showed apoptosis of the cells in addition to caspase-3 activation and PARP cleavage. Moreover, EGCG suppressed Bcl-2, XIAP and Mcl-1 [355].

A recent study demonstrated that targeting Axl RTK by a Src/Abl kinase inhibitor, bosutinib (SKI-606) or a specific-inhibitor of Axl (R428), leads to a potent induction of CLL cell apoptosis in a dose- and time dependent manner [354].

As far as ROR1 is considered there is only one study published with regard to targeted therapy of this RTK in CLL. Recently, a report has described the generation of a panel of mAbs in chimeric rabbit/human Fab and IgG1 formats selected by phage display. These antibodies were tested for their ability to mediate ADCC, CDC and internalization. The mAb with the highest affinity and slowest rate of internalization was the only mAb that mediated a weak ADCC, however no CDC or direct apoptosis induction was detected by these mAbs. Furthermore, the internalization data suggested that ROR1 might a suitable target for the delivery of cytotoxic loads by antibody-drug conjugates and immunotoxins [330].

This thesis includes a study (paper III) where we generated five mouse mAbs against peptides of extracellular Ig, CRD and KNG domains of ROR1. ROR1 mAbs could specifically recognise ROR1 protein on surface of CLL cells. Three functional assays i.e. direct induction of apoptosis, cross-linking of the surface bound ROR1 mAbs as well as complement in lysis were utilized and compared with murine and chimeric anti-CD20 (rituximab) mAbs, respectively. ROR1 mAbs were capable of inducing apoptosis using all the three functional assays. Different antibodies varied in their percentages of apoptosis, these findings are discussed extensively in the result and discussion sections. There was no clear correlation between ROR1 expression and apoptosis. Moreover, antibodies alone against the CRD and KNG regions had the highest cytotoxic capability. In conclusion we targeted CLL cells with specific antibodies against different regions of extracellular domain of ROR1 and by doing so, our ROR1 mAbs induced apoptosis and this resulted in cleavage of PARP. Furthermore, by producing fully human ROR1 mAbs capable of killing CLL cells, we might be able to look for a new way of targeted therapy of CLL. Additionally, our preliminary results indicate that ROR1 specific small inhibiting molecules are effective in apoptosis induction in primary CLL cells (data to be published). In summary, mAb therapy and tyrosine kinase inhibition might be possible targeted therapies in CLL.

Table 6. Therapeutic monoclonal antibodies used in oncology

Generic name (trade name; sponsoring companies)	Target	Antibody Format	Cancer Indication
Unconjugated antibodies			
Rituximab (Rituxan/Mabthera; Genentech/Roche/Biogen Idec)	CD20	Chimeric IgG1	Non-Hodgkin lymphoma
Trastuzumab (Herceptin; Genentech/ Roche)	HER2	Humanized IgG1	Breast cancer
Alemtuzumab (Campath/ MabCampath; Genzyme/Bayer)	CD52	Humanized IgG1	Chronic lymphocytic leukaemia
Cetuximab (Erbitux; ImClone Systems/Bristol–Myers Squibb)	EGFR	Chimeric IgG1	Colorectal cancer
Bevacizumab (Avastin; Genentech)	VEGFA	Humanized IgG1	Colorectal, breast and lung cancer
Panitumumab (Vectibix; Amgen)	EGFR	Human IgG2	Colorectal cancer
Ofatumumab (Arzerra; Genmab/ GlaxoSmithKline)	CD20	Human IgG1	Chronic lymphocytic leuakemia
Immunoconjugates			
Gemtuzumab ozogamicin (Mylotarg; Pfizer)	CD33	Humanized IgG4	Acute myelogenous leukaemia
^{90}Y-Ibritumomab tiuxetan (Zevalin; Biogen Idec)	CD20	Mouse	Lymphoma
Tositumomab and ^{131}I-tositumomab (Bexxar; GlaxoSmithKline)	CD20	Mouse	Lymphoma

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4 AIMS OF THE THESIS

Paper I
To investigate the expression of ROR1 in CLL and normal controls.

Paper II
To study the effects of siRNA silencing of ROR1 and FMOD in CLL cells.

Paper III
To generate mouse anti-ROR1 monoclonal antibodies inducing apoptosis of CLL cells.

Paper IV
To study ROR1 and ROR2 expression in hematological malignancies of lymphoid and myeloid origins.

5 RESULTS

PAPER I

ROR1, a cell surface receptor tyrosine kinase is expressed in chronic lymphocytic leukemia and may serve as a putative target for therapy

Int J Cancer. 2008, 123(5):1190-95

Gene profiling studies in 2001 showed downregulations and upregulations of many genes in CLL, among them a 43.8-fold increase of ROR1 in CLL cells. This study was desgined to investigate the expression pattern of ROR1 in CLL and healthy donors both at the gene and protein levels. ROR1 is a member of the receptor tyrosine kinase (RTK) family. ROR1 receptors are cell surface receptors participating in signal transduction, cell–cell interaction, regulation of cell proliferation, differentiation, cell metabolism and survival. ROR1 is highly conserved between species, e.g. human, mouse, drosophila and C.elegans suggesting important biological functions and is normally expressed in heart, lung and kidneys in adult humans and to a lesser extent in the placenta, pancreas and skeletal muscles.

PBMC of all CLL patients (n=100) expressed ROR1 at the mRNA level, no expression of ROR1 was detected in healthy donor PBMC (n=10), normal B cells (n=6), normal T cells (n=3), or enriched blood granulocytes (n=10).

Protein expression of ROR1 was analyzed by Western blot as well as flow cytometry. Our N-terminal and C-terminal ROR1 antibodies detected two ROR1 specific bands of 105 and 130 kDa in CLL samples (n=18). ROR1 commercial antibody detected only the 105 kDa band. No band was seen in healthy controls (n=10).

Using ROR1 commercial polyclonal antibody, surface expression of ROR1 in progressive (n=9) and non-progressive (n=9) was detected by flow cytometry. The frequency of $CD19^+$ CLL cells expressing ROR1 varied 71 ± 5% (mean ± SEM) (range: 36–92%). Healthy donor B cells (n=10) did not express ROR1.

PMA/ionomycin could not further activate CLL cells and tonsil B cells due to their constitutive ROR1 mRNA expression but induced gene expression of ROR1 in normal B and T cells.

Mutation analysis of cloned extracellular and cytoplasmic kinase domains of the ROR1 gene was performed in 10 CLL patients and showed no major genomic aberrations. FISH analysis of PBMC from 3 CLL patients showed no rearrangement in the 1 p region. Bone marrow cell analysis from 4 CLL patients showed no abnormality at the ROR locus.

In conclusion our results indicate that ROR1 is overexpressed in CLL patients both at gene and protein leves but not in healthy donors. RTKs might be interesting targets for cancer therapy and ROR1 could be a potential candidate structure for targeted therapy using monoclonal antibodies and small inhibiting molecules.

PAPER II

Silencing of ROR1 and FMOD with siRNA results in apoptosis of CLL cells

Br J Haematol. 2010, 151(4):327-35

The receptor tyrosine kinase ROR1 and small leucine rich proteoglycan Fibromodulin (FMOD) are upregulated in chronic lymphocytic leukaemia (CLL) cells. Small interfering RNA (siRNA) can specifically interfere with the expression of any given gene. In this paper we wanted to see if siRNAs transfection of CLL cells will lead to downregulation of ROR1 and FMOD gene and protein levels and if so what would be the functional effect of this downregulation.

The real-time PCR results demonstrated that the transfection of CLL cells with siRNA constructs silenced ROR1 and FMOD expression by (75–95%). There was no downregulation of the housekeeping gene after transfection period of 24 h.

After 48 h of siRNA treatment, Western blot analysis demonstrated that ROR1 and FMOD proteins were significantly downregulated. The corresponding 105 kDa and 59 kDa bands of ROR1 and FMOD were absent in CLL cells transfected with *ROR1*-specific siRNA as compared to untransfected and control siRNA- transfected CLL cells.

Silencing of FMOD and ROR1 resulted in a statistically significant apoptosis of CLL cells but not of healthy donors PBMC (enriched B and T cells). Time kinetics indicated that apoptosis was noted as early as 4 h after transfection and was continuing at the end of 48 h.

Four $FMOD^{+}$ $ROR1^{+}$ human fibroblast cell lines were tested for apoptosis induction following siRNA transfections. Only one of these cell lines, HFFF-PI 6 demonstrated induction of apoptosis.

In summary we have shown that ROR1 and FMOD could be downregulated at gene and protein levels by specific ROR1 and FMOD siRNAs. This downregulation resulted in induction of apoptosis of ROR1 and FMOD but not healthy B and T cells. These results suggest that ROR1 and FMOD are implicated as factors for survival of CLL cells.

PAPER III

Monoclonal antibodies against ROR1 induce apoptosis of chronic lymphocytic leukemia (CLL) cells. (Submitted)

In this paper the aim was to generate monoclonal antibodies against the extracellular domains of ROR1 capable of detecting surface expression of ROR1. Our intention was to have functional ROR1 antibodies to test apoptotic effects of these monoclonal antibodies on CLL cells in vitro. ROR1 is highly conserved in human, mouse, Drosophila and C. elegans suggesting crucial biological fuctions. ROR1 consists of an extracellular part including an immunoglobulin (Ig) -like domain, a cystein-rich domain (CRD) and a kringle (KNG) domain as well as an intracellular tyrosine kinase and a proline-rich domain. ROR1 is constitutively phosphorylated in CLL. ROR1 siRNA transfection also induced specific apoptosis of CLL cells.

Five ROR1 monoclonal antibodies were raised against extracellular domains of ROR1. One was against the Ig domain, Ig (3B8) MAb, two against the CRD domain, CRD (1C11) and CRD (1D8) MAbs and two against the KNG domain, KNG (4C10) and KNG (4A7) MAbs. For specificity controls HeLa cells were transfectred with ROR1 gene construct plasmid. In Western blot all five MAbs recognized specifically a band of 100-105 kDa. No band was detected in untransfected cells.

ROR1 surface expression was detected by flow cytometry using all 5 ROR1 monoclonal antibodies in CLL patients (n=20). The frequency of $ROR1^+$ /$CD19^+$/$CD5^+$ cells for each of the ROR1 monoclonal antibodies were as the following. Ig (3B8): 92 ± 1% (mean±SEM) (range: 77-98%); CRD (1C11): 94 ± 1% (72-98%); (CRD (1D8): 88 ± 2% (72-94%); (KNG (4A7): 93 ± 1% (74-98%); KNG (4C10): 94 ± 1% (84-99%). No expression of ROR1 in B cells of healthy donors was detected. Patients with progressive disease demonstrated a significantly higher proportion of $ROR1^+$ CLL cells as compared to non-progressive patients. Ig (3B8), CRD (1C11) and KNG (4A7) MAbs stained a significantly higher ($p<0.05$ – $p<0.01$) frequency of $ROR1^+$ cells in unmutated CLL patients compared to the mutated CLL patients. This was not the case for the other two ROR1 MAbs.

Apoptosis induction was measured by Annexin V assay in PBMC from CLL patients (n=20) and healthy donors (n=8) using all five ROR1 MAbs. Based on time-kinetics experiments the 18 h incubation time was chosen for incubation of antibodies with CLL and healthy cells. All ROR1 MAbs alone induced apoptosis of the CLL cells of all patients. The differences compared to CLL cells with no antibody or isotype controls were statistically significant ($p<0.0001$). No apoptosis of normal PBMC was detected. CRD (1C11), CRD (1D8) and KNG (4C10) MAbs were the best to induce apoptosis of CLL cells. These three MAbs (1C11, 1D8, 4C10) were also more effective in apoptosis induction than the murine (LT-20) or the chimeric (rituximab) anti-CD20 MAb ($p<0.0001$). There was no relation between the frequency of $ROR1^+$ CLL cells and direct apoptosis.

Cross-linking of the anti-ROR1 MAbs using F(ab´)2 fragments of anti-Fc antibodies significantly augmented apoptosis. Two of the MAbs induced complement-dependent cytotoxicity similar to that of rituximab.

Western blot analysis indicated PARP protein cleavage of CLL cell lysates undergoing apoptosis. All five anti-ROR1 MAbs induced a PARP cleavage product of 85 kDa.

In summary, we describe the generation, staining pattern and cytotoxicity of a panel of mouse MAbs against ROR1. The identified functional epitopes of ROR1 in CLL will be further utilized in the development of human antibodies against ROR1 for clinical trials.

PAPER IV

The receptor tyrosine kinases ROR1 and ROR2 expression in hematologic malignancies. (Manuscript)

In this study ROR1and ROR2 expression in hematological malignancies of lymphoid and myeloid origins was investigated. ROR1 monoclonal antibodies were raised against the extracellular part of ROR1 and commercially available ROR1 and ROR2 polyclonal antibodies were used for this study. ROR1 expression pattern during progression of the CLL disease was also studied.

ROR1 and ROR2 are cell surface receptors, containing an immunoglobulin-like (Ig) domain, a cysteine-rich (CRD) domain and a kringle domain (KNG) in their extracellular portion. The intracellular region consists of a kinase-like domain followed by alternating regions of serine-threonine and proline-rich motifs. ROR1 is considered a potent survival kinase and of importance during embryogenesis and organogenesis. ROR1 is not expressed in normal adult lymphoid and non-lymphoid tissues with the exception for adipose tissue. ROR2 is involved in the early formation of the chondrocytes and important for cartilage and growth plate development. ROR2 deficiency results in 2 skeletal abnormalities, Robinow Syndrome and Brachydactyly Type B. No such abnormalities are reported for ROR1.

The results of this study showed a statistically significant variation in the expression of ROR1 in various hematological malignancies as compared to controls (no expression of ROR1 in PBMC of healthy donors or “reactive” lymph nodes). In flow cytometry, 24/24 of chronic lymphocytic leukemia (CLL) cases were positive for ROR1 surface expression range of 79-91%, 9/9 mantle cell lymphomas (MCL) (13-93%), 1/1 hairy cell leukemia (HCL) (91%), 8/8 marginal zone lymphoma (MZL) (28-76%), 11/11 diffuse large B-cell lymphoma (DLBCL) (30-81%), 20/30 follicular lymphoma (FL) (1-89%), 18/23 acute lymphoblatic leukemia (ALL) (1-87%), 19/24 acute myelogenous leukemia (AML) (1-95%), 14/14 chronic myelogenous leukemia (CML) (22-80%) and 14/14 multiple myeloma (MM) (36-83%).

Two cell lines reported to be positive for ROR2, K-562 (a Human erythromyeloblastoid leukemia cell line) and HeLa (a cervical cancer cell line) were used as controls. No expression of ROR2 was detected in hematological malignancies. PBMC from healthy donors did not show any expression of ROR1 and ROR2. A statistically significant higher expression of ROR1 was detected in progressive compared to non-progressive CLL patients (n=7). ROR1 was shown to be stable overtime in stable CLL patients (n=5).

In summary we report the surface expression of receptor tyrosine kinase (ROR1) in all hematological malignancies tested, both of lymphoid and myeloid origins. No expression of ROR1 and ROR2 was detected in normal donors and reactive lymph nodes. Non-progressive and progressive patients showed a statistically significant difference in expression of ROR1. ROR1 expression was constant during the course of CLL disease. Own generated monoclonal antibodies detected a significantly higher expression of ROR1 than commercial polyclonal antibodies.

6 DISCUSSION

6.1 ROR1 CHARACTERIZATION

Gene expression profiling of CLL cells has revealed downregulations and upregulations of hundreds of genes with various chromosomal localizations. These gene transcripts were more prominent in CLL samples compared to other B-cell malignancies and normal B cells. However the mutated and unmutated CLL cases showed equal abundance of the gene transcripts [327, 359]. Among upregulated genes in CLL, there was a 43.8-fold increase of the orphan receptor tyrosine kinase (RTK) ROR1. ROR1 is a transmembrane protein located in plasma membrane with a protein size of 105 kDa, localized at chromosome 1p31.3 and its structure consists of an extracellular region, a single transmembrane region and an intracellular region [300]. It is interesting to know that vascular endothelial growth factor receptor (VEGFR) was the first RTK reported in CLL [351, 352] and ROR1 the subsequent [269-271].

Surprisingly, the ligand for ROR1 was not known until recently and hence the nomenclature, orphan receptor tyrosine kinases (ROR1 and ROR2). Studies demonstrated that Wnt molecules such as Wnt5a might be a possible ligand for ROR1/ROR2 by interacting with the ligand binding motifs of cysteine-rich (CRD) and kringle (KNG) domains of ROR family members [271, 317], further suggesting that ROR1/ROR2 could act as receptors for Wnt family proteins [305].

Wnt signaling pathways are reported to have a vital role in cellular processes such as cell-to-cell interaction, differentiation and morphogenesis in embryonic development. Deregulation or aberrant function of Wnt signaling is found in many types of cancer, developmental defects and inherited diseases [360]. ROR1/ROR2 are reported to have a crucial function in the non-canonical Wnt signaling [299, 314, 317].

The extracellular domain of ROR1 could bind Wnt5a molecule and coexpression of ROR1 and Wnt5a in HEK cells lead to the activation of NF-κB. Additionally, CLL cells viability cocultured with Wnt5a-expressing Chinese Hamster Ovary (CHO) cells was significantly greater than those cultured with CHO cells alone. This survival advantage was neutralized by the addition of antisera against ROR1 [249].

Studies have shown that several Wnt genes are highly expressed in CLL [361] and high levels of Wnt5a secretion have also been detected in several hematological malignancies [362, 363]. Stromal cells producing Wnt proteins support the survival of CLL B cells [364]. Furthermore, Wnt proteins secreted by both cancer cells and stromal cells, might act as growth factors for malignancies in an autocrine or paracrine manner [365, 366]. It may be postulated that Wnt5a might be a growth and survival factor for CLL supporting survival of leukemic B cells.

ROR1 signaling events are poorly understood, but there are reports showing Wnt binding to ROR1/ROR2 receptors results in a sequence of molecular events, involving ROR phosphorylation, activation of PI3K, Cdc42 and MKK7, further leading to cytoskeletal changes and/or JNK-mediated phosphorylation of the transcription factor

ATF2 [367-369]. Besides, other putative components of the ROR1/non-canonical Wnt pathway, specially Vangl2, and Dvl2 were reported to be involved with ROR1 expression in CLL [370].

Moreover, it is known that STAT3 is constitutively phosphorylated in CLL [371]. Phosphorylated STAT3 binds to promoter regions of ROR1 and VEGFR leading to activation and by so inducing production of ROR1 and VEGFR proteins in CLL [355, 372]. STAT3 could also induce expression of Wnt5a [342]. STAT3 activation is reported for various solid and hematologic tumors and related to anti-apoptotic mechanisms and growth stimulation of the malignancies [373].

ROR1 is spontaneously expressed at the gene and protein levels of the leukemic cells in all CLL patients but not in normal peripheral blood leukocytes (paper I) and this was further supported by other reports [269, 271]. Additionally a uniform high expression in both IGHV mutated and unmutated CLL cases, as well as in the non-progressive (NP) and progressive (P) phase of the disease was detected using a commercial pAb in flow cytometric analyses (paper I). Furthermore, using own anti-ROR1 mAbs surface expression of ROR1 indicated a higher frequency of ROR1 in (P) than (NP) as well as in unmutated than mutated cases, respectively (paper III). In contrary to others showing no difference with (P) and (NP) disease [269, 332], we defined a clear difference between two subgroups of CLL patients with regard to ROR1 expression. One explanation for this might be they used anti-ROR1 pAb and in one of the studies [332] the antibody was conjugated giving a high frequency of staining of CLL cells (paper III). Moreover, own anti-ROR1 mAbs showed a statistically higher expression of ROR1 compared to the commercially available pAbs (paper IV). Collectively, these results together with a recent publication [332] might propose a diagnostic and/or prognostic role for ROR1 in CLL. In addition, the level of surface expression of ROR1 and the absence in normal blood leukocytes indicates that ROR1 might be a candidate structure for further evaluation for targeted therapy.

Protein analyses by Western blots using pAbs against the N-terminal part of ROR1 revealed a single band of 105 kDa (the full-length transcript of ROR1) in CLL patients [300, 374]. Contrary to the N-terminal ab, a C-terminal ab revealed 2 bands of 105 and 130 kDa (paper I). Altered glycosylation might explain the difference. The 130 kDa band may represent the fully glycosylated variant of ROR1 and 105 the unglycosylated protein [375]. Posttranslational modifications (PTMs) are processes important for proper functioning of many proteins with glycosylation, phosphorylation and ubiquitination among the important PTMs of proteins. Recently, a publication has confirmed the presence of the glycosylated 130 kDa band in CLL. In addition it was found that ROR1 is posttranslationally modified by glycosylation and mono-ubiquitination. These modifications regulate ROR1 localization and signaling and are highly variable among individual CLL patients [370].

In addition to glycosylation, phosphorylation of ROR1 is probably one of the important PTMs regulating the functional activity of ROR1 in the CLL cells. Phosphorylation of RTKs, especially at tyrosine residues is a major mechanism for regulation of different cellular processes including cell division, protein synthesis and transcriptional regulation [299, 376].

The preliminary observation that ROR1 is constitutively phosphorylated in CLL (data to be published) indicates that this RTK might be involved in the pathobiology of CLL. Additionally a significant correlation was found between progressive stage of CLL and phosphorylation of ROR1 (data to be published).

Further investigation of the trio, Wnt signaling proteins-specially Wnt5a, ROR receptors-particularly ROR1 and STAT proteins-mainly STAT3, seems to be a logical approach to know the hidden aspects of their interaction with one another, to decode the unknown events of ROR1 signaling pathway and to understand the impact this might have on CLL pathobiology.

6.2 FUNCTIONAL STUDIES OF ROR1

The discovery of molecules uniquely overexpressed by malignant cells and critical for the growth and survival of the cancerous cells are crucial in defining new therapeutic strategies in human cancers. Dysregulation of kinases and phosphatases, which under normal conditions control the reversible process of phosphorylation, occurs in many diseases including cancer.

The functional role of the kinase and phosphatase component of the human genome in apoptosis is poorly understood and entails the identification of new cell survival regulators and drug targets. For this purpose and to identify these kinases and phosphatases crucial to the survival of cancer cells, a high-throughput RNAi screening was utilized [377]. In the screen, 650 known and putative kinases were targeted by RNAi in HeLa cervival carcinoma cells. Of the 73 genes that were overall identified as survival kinases, ROR1 was one of four most potent. A greater than three fold increase in apoptosis was noted in HeLa cells, 72 h after silencing of ROR1.

Based on the results from the RNAi study, we wanted to investigate the role of ROR1 in the survival of CLL cells by using siRNAs specifically silencing the gene (paper II). The treatment of CLL cells by specific ROR1 siRNAs induced reduction of ROR1 mRNA expression. Additionally, Western blot analysis demonstrated that ROR1 protein was also downregulated 48 h after siRNA treatment. The outcome of silencing of ROR1 was a statistically significant apoptosis of CLL cells but not of B cells from normal donors. Overall our results of siRNA downregulation of ROR1 in CLL cells together with the RNAi screening, suggested that ROR1 is an important survival kinase in cancer and can be utilized as a target for therapeutic approaches.

The current mAb therapy of CLL includes rituximab (anti-CD20), ofatumumab (anti-CD20) and alemtuzumab (anti-CD52) [378]. Even though antibody therapy increases progression-free survival and overall survival of CLL patients, the widespread expression of these antigens on normal B, T and NK cells in addition to monocytes, may elicit complications such as hepatitis B virus reactivation and cytomegalovirus (CMV) reactivation following rituximab and alemtuzumab administration, respectively [379, 380].

The important features of surface antigens for a successful mAb therapy include a restricted, uniform and constitutive cell surface expression [381, 382]. Additionally, three out of eleven mAbs approved for cancer therapy are indeed used in CLL treatment [383],

indicating that CLL is a preferred field with regard to antibody therapy [198]. Furthermore, CLL is a disease with availability of leukemic cells in circulation and also accessibility of mediators of antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). The structure of ROR1, consisting of extracellular and intracellular regions, also helps in designing targeted therapies in the form of antibody therapy or small molecule inhibitors.

ROR1 appeared to meet most of these criteria, as a restricted and constitutive high expression was detected in all CLL patients tested with no expression of ROR1 in normal peripheral blood leukocytes [384]. Moreover, these results gave us a strong incentive for generating anti-ROR1 monoclonal antibodies for diagnostic purposes and the first step towards targeted therapy of CLL.

For the same reason and in order to explore functionally suitable ROR1 epitopes for generation of human ROR1 mAbs for clinical trials and also to investigate the apoptotic effects of monoclonal antibodies on CLL cells in vitro, five mouse mAbs were generated.

These mAbs were raised against peptides of extracellular Ig, CRD and KNG domains of ROR1. Interestingly, individual CLL patients showed a variance in the staining pattern by the different ROR1 mAbs. This might suggest a structural heterogeneity in the ROR1 expression. Studies have shown a direct correlation between increased expression of RTK on tumor cells to higher proliferative potential and/or survival advantage of the tumor cells, indicating a poor clinical prognosis as well [215, 236]. In our study we observed that CLL patients follow the same pattern, with progressive patients having a higher frequency of ROR1 positive cells than those with non-progressive disease. A similar higher expression of ROR1 occurred in patients with unmutated IGHV genes compared to the mutated ones.

Three functional assays i.e. direct induction of apoptosis, cross-linking of the surface bound ROR1 mAbs as well as complement in lysis were utilized and compared with murine and chimeric anti-CD20 (rituximab) mAbs, respectively. ROR1 mAbs were capable of inducing apoptosis. Different antibodies varied in their percentages of apoptosis, these were discussed in result section. There was no clear correlation between ROR1 expression [(frequency and density) (MFI)] and apoptosis (data not shown) and a similar phenomenon has been reported for mAbs against CD20 and RTKs [277, 385]. Moreover, antibodies alone against the CRD and KNG regions had the highest cytotoxic capability. This is an interesting observation as the CRD and KNG regions are presumed to contain Wnt family and non-Wnt-ligand binding modules involved in diverse cellular responses [11, 299, 305, 386].

These results provide the groundwork to further study ROR1 characterization in CLL patients and also calls for strategies to develop human mAbs that selectively target ROR1 in CLL. It is predicted that such mAbs would facilitate a precise intervention without associated immune suppression.

In addition to mAb therapy, ROR1 has also been shown to undergo apoptosis when targeted with specific small RTK inhibitor molecules (own unpublished data). In a recent study, CD8+ T cells have been engineered to express a ROR1-specific chimeric antigen receptor (CAR) that are able to recognize and lyse primary B CLL cells, but not normal B cells in vitro, suggesting the role of ROR1 molecule as a TAA and also using ROR1-

specific T cell in treatment of CLL patients [321]. The role of ROR1, as a putative tumor associated antigen in active immunotherapy of CLL patients, has not been identified yet. In this regard and to explore the functional activity of CLL T cells against ROR1 molecule, the differences in the frequencies of activated T cells producing IFN-γ, IL-5, Il-17A and TGF-β in response to ROR1 peptides, in non-progressive versus progressive and IGHV mutated versus unmutated CLL patients, were investigated and compared to that of healthy controls (data to be published).

6.3 ROR AND HEMATOLOGICAL MALIGNANCIES

ROR family members (ROR1 and ROR2) have a similar structure consisting of a ligand binding extracellular part, a transmembrane part and an intracellular part with tyrosine kinase activity. Moreover, ROR1 and ROR2 are parts of the Wnt signaling pathway [305] and important for organogenesis during the embryonic stage [300, 301, 322, 325].

Additionally, human ROR1 and ROR2 genes (hRor1 and hRor2) share an overall 58% identity in their amino acid sequence [300]. Furthermore previous publications have shown the gene expression of ROR1 in acute lymphocytic leukemia (ALL) patients [328, 329] and recently at the protein level in different types of non-Hodgkin lymphomas (NHL) [321, 330-332] and in several solid tumors [333, 334].

ROR2 on the other hand has been shown to be overexpressed in several solid tumors such as squamous cell carcinoma [345], malignant melanoma [346], osteosarcoma [347], renal cell carcinoma [348], prostate and gastric cancers [349, 350]. ROR2 was not detected in CLL and white blood cells of normal donors [269]. Mutations in the human ROR2 gene cause two well characterized skeletal defects, recessive Robinow syndrome (RRS) [335, 336] and dominant brachydactyly B (BDB) [337, 338].

Since there were no reports of ROR1 in malignancies of myeloid origins and also the fact that the expression pattern of ROR2 in hematological malignancies has not been investigated, we designed a study to find out more about the expression of ROR1 and ROR2 in hematological malignancies of lymphoid and myeloid origins. Our results indicated a significant variation in the expression of ROR1 in various hematological malignancies including CLL, MCL, MZL, DLBCL, FL, B-ALL, AML, CML and MM. Interestingly we could not detect ROR2 expression in any of the malignancies tested including CLL. There were no expressions of ROR1 and ROR2 in in PBMC of healthy donors.

The exact reason for expression of ROR1 but not ROR2 in hematological malignancies is not known. One explanation might be the connection between Gamma interferon activation site (GAS) elements, STAT3 and ROR1 in hematological malignancies including CLL. Gamma interferon activation site (GAS) elements are short stretches of DNA-recognition motif in the promoter region of various genes [387]. It is reported that STAT3 activation results in binding of STAT3 to promoter region of target genes containing these DNA-recognition motifs (GAS elements) dramatically increasing the transcription of these genes [373]. We know that STAT3 is constitutively phosphorylated in CLL as well as in other hematologic malignancies [371, 373]. ROR1 promoter region

contain these GAS elements [372] while there are no reports indicating this for ROR2. Phosphorylated STAT3 by binding to the ROR1 promoter region (GAS elements) may cause activation of ROR1 leading to ROR1 protein production [372]. However, there are no reports indicating an association between STAT3, GAS elements and ROR2 in cancer.

Another explanation might be that the level of hypo and hypermethylation in the promoter regions of ROR1 and ROR2 could be different. It is known that many kinases might be switched on or off by intracellular oxidation process. There is also a possibility that ROR1 activation could be triggered by reactive oxygen species (ROS) as has been shown for Axl activation in CLL [388]. CLL B cells compared to normal lymphocytes have increased reactive oxygen species [389].

7 CONCLUSIONS AND FUTURE PERSPECTIVES

Aberrant tyrosine kinase activation may increase the survival, proliferation and drug resistance of malignant cells. More than thirty of known receptor tyrosine kinases (RTKs) are implicated in different human malignancies [200, 219]. A gene profiling study pointed the way towards a connection between the ROR1 RTK and CLL [327].

The results presented in this thesis can be divided into two main sections, characterization of ROR1 and ROR2 in CLL and other hematological malignancies (paper I and IV). Functional studies utilizing siRNA and mAbs for downregulation of the functional activity and apoptosis of CLL cells (paper II and III).

Firstly we were able to show that ROR1 is overexpressed in CLL patients both at gene and protein levels but not in healthy donors. Next we reported a significant variation in the expression of ROR1 in a panel of hematological malignancies tested, both of lymphoid and myeloid origins. Contrary to ROR1, ROR2 was not detected in hematological malignancies tested. ROR1 expression was related to the activity of the disease. No expression of ROR1 and ROR2 were noted in PBMC of healthy donors.

Second part of results of this thesis focused on two functional studies of ROR1, siRNA downregulation and in vitro antibody treatment of leukemic B CLL cells. It was demonstrated that ROR1 could be downregulated at gene and protein levels by specific ROR1 siRNAs. Downregulation of ROR1 resulted in induction of apoptosis of ROR1 in CLL cells but not in healthy B and T cells, thus identifying ROR1 as a survival kinase in CLL cells. Moreover, we generated a panel of mouse anti-ROR1 mAbs against extracelluar regions of ROR1 including immunoglobulin (Ig), cysteine-rich (CRD) and kringle (KNG) domains.

These mAbs were capable of specifically recognizing cell surface expression of ROR1 in CLL patients to a varying degree. Patients with progressive disease demonstrated a significantly higher proportion of ROR1 positive CLL cells as compared to non-progressive patients. Furthermore, a significantly higher ($p<0.05$ – $p<0.01$) frequency of ROR1 positive cells was detected in unmutated CLL patients compared to the mutated CLL patients. These antibodies were also able to induce apoptosis of leukemic B cells of CLL patients in vitro compared to murine and chimeric anti-CD20 (rituximab) mAbs. Interestingly, anti-ROR1 CRD and KNG mAbs exhibited higher apoptosis induction, this was of particular importance as CRD and KNG regions are potential ligand binding sites of RTKs. Additionally, no relation between the frequency of ROR1 positive CLL cells and direct apoptosis was found.

There is a constant need to improve therapy of CLL, especially with regard to mAb therapies, as such approaches seem to be safe and effective choices for treatment. We expect and foresee the generation and development of human antibodies against ROR1 for clinical trials using identified functional epitopes of ROR1 in CLL. Moreover, ROR1 therapy may not only be restricted to CLL, in fact it has the potential of targeting other hematological malignancies as well as solid tumors. Furthermore, unique structure of ROR1 with an active kinase domain allows for other forms of targeted therapies such as

small RTK inhibitor molecules to be applied in future ROR1 positive malignancies. In addition to all the targeted therapeutic options, ROR1 can be potentially used in clinics for diagnosis and/or prognosis of CLL in near future.

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9 REFERENCES

1. Zenz, T., et al., From pathogenesis to treatment of chronic lymphocytic leukaemia. Nat Rev Cancer, 2010. 10(1): p. 37-50.
2. Chiorazzi, N., K.R. Rai, and M. Ferrarini, Chronic lymphocytic leukemia. N Engl J Med, 2005. 352(8): p. 804-15.
3. Ternynck, T., et al., Comparison of normal and CLL lymphocyte surface Ig determinants using peroxidase-labeled antibodies. I. Detection and quantitation of light chain determinants. Blood, 1974. 43(6): p. 789-95.
4. Matutes, E., et al., The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia, 1994. 8(10): p. 1640-5.
5. Moreau, E.J., et al., Improvement of the chronic lymphocytic leukemia scoring system with the monoclonal antibody SN8 (CD79b). Am J Clin Pathol, 1997. 108(4): p. 378-82.
6. Foon, K.A. and M.J. Hallek, Changing paradigms in the treatment of chronic lymphocytic leukemia. Leukemia, 2010. 24(3): p. 500-11.
7. Dighiero, G. and T.J. Hamblin, Chronic lymphocytic leukaemia. Lancet, 2008. 371(9617): p. 1017-29.
8. Tam, C.S. and M.J. Keating, Chemoimmunotherapy of chronic lymphocytic leukemia. Nat Rev Clin Oncol, 2010. 7(9): p. 521-32.
9. Ghia, P., A.M. Ferreri, and F. Caligaris-Cappio, Chronic lymphocytic leukemia. Crit Rev Oncol Hematol, 2007. 64(3): p. 234-46.
10. Rozman, C. and E. Montserrat, Chronic lymphocytic leukemia. N Engl J Med, 1995. 333(16): p. 1052-7.
11. Reddy, U.R., S. Phatak, and D. Pleasure, Human neural tissues express a truncated Ror1 receptor tyrosine kinase, lacking both extracellular and transmembrane domains. Oncogene, 1996. 13(7): p. 1555-9.
12. http://ocsyd.se/VP-verksamhet/Kvalitetsreg/RappotLymfom2000-2006.pdf.
13. Dores, G.M., et al., Chronic lymphocytic leukaemia and small lymphocytic lymphoma: overview of the descriptive epidemiology. Br J Haematol, 2007. 139(5): p. 809-19.
14. http://seer.cancer.gov/csr/1975_2006/.
15. Gribben, J.G., How I treat CLL up front. Blood, 2010. 115(2): p. 187-97.
16. De Lima, M., et al., Chronic lymphocytic leukemia in the young patient. Semin Oncol, 1998. 25(1): p. 107-16.
17. Boelens, J., et al., Chronic lymphocytic leukaemia. Anticancer Res, 2009. 29(2): p. 605-15.
18. Weiss, N.S., Geographical variation in the incidence of the leukemias and lymphomas. Natl Cancer Inst Monogr, 1979(53): p. 139-42.
19. Boggs, D.R., et al., Chronic lymphocytic leukemia in China. Am J Hematol, 1987. 25(3): p. 349-54.
20. Yuille, M.R., et al., Familial chronic lymphocytic leukaemia: a survey and review of published studies. Br J Haematol, 2000. 109(4): p. 794-9.
21. Zent, C.S., et al., Chronic lymphocytic leukemia incidence is substantially higher than estimated from tumor registry data. Cancer, 2001. 92(5): p. 1325-30.
22. Linet, M.S., et al., Familial cancer history and chronic lymphocytic leukemia. A case-control study. Am J Epidemiol, 1989. 130(4): p. 655-64.
23. Neuland, C.Y., et al., Familial chronic lymphocytic leukemia. J Natl Cancer Inst, 1983. 71(6): p. 1143-50.
24. Capalbo, S., et al., Increased risk of lymphoproliferative disorders in relatives of patients with B-cell chronic lymphocytic leukemia: relevance of the degree of familial linkage. Eur J Haematol, 2000. 65(2): p. 114-7.

25. Ishibe, N., et al., Clinical characteristics of familial B-CLL in the National Cancer Institute Familial Registry. Leuk Lymphoma, 2001. 42(1-2): p. 99-108.
26. Anderson, L.A., et al., Survival patterns among lymphoma patients with a family history of lymphoma. J Clin Oncol, 2008. 26(30): p. 4958-65.
27. Wiernik, P.H., et al., Anticipation in familial chronic lymphocytic leukaemia. Br J Haematol, 2001. 113(2): p. 407-14.
28. Horwitz, M., E.L. Goode, and G.P. Jarvik, Anticipation in familial leukemia. Am J Hum Genet, 1996. 59(5): p. 990-8.
29. Goldin, L.R., et al., Anticipation in familial chronic lymphocytic leukemia. Am J Hum Genet, 1999. 65(1): p. 265-9.
30. Hallek, M., et al., Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood, 2008. 111(12): p. 5446-56.
31. Melo, J.V., D. Catovsky, and D.A. Galton, The relationship between chronic lymphocytic leukaemia and prolymphocytic leukaemia. I. Clinical and laboratory features of 300 patients and characterization of an intermediate group. Br J Haematol, 1986. 63(2): p. 377-87.
32. Ginaldi, L., et al., Levels of expression of CD19 and CD20 in chronic B cell leukaemias. J Clin Pathol, 1998. 51(5): p. 364-9.
33. Cheson, B.D., et al., National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood, 1996. 87(12): p. 4990-7.
34. Cramer, P. and M. Hallek, Prognostic factors in chronic lymphocytic leukemia-what do we need to know? Nat Rev Clin Oncol, 2011. 8(1): p. 38-47.
35. Muller-Hermelink, H.K., et al., Chronic lymphocytic leukaemia/small lymphocytic lymphoma. In: Jaff e ES, Harris NL, Stein H, Vardiman JW, eds. World Health Organization classification of tumours: pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon: IARC Press, 2001: 127–30. 2001.
36. Rawstron, A.C., et al., Monoclonal B lymphocytes with the characteristics of "indolent" chronic lymphocytic leukemia are present in 3.5% of adults with normal blood counts. Blood, 2002. 100(2): p. 635-9.
37. Ghia, P., et al., Monoclonal CD5+ and CD5- B-lymphocyte expansions are frequent in the peripheral blood of the elderly. Blood, 2004. 103(6): p. 2337-42.
38. Rawstron, A.C., et al., Inherited predisposition to CLL is detectable as subclinical monoclonal B-lymphocyte expansion. Blood, 2002. 100(7): p. 2289-90.
39. Marti, G.E., et al., Diagnostic criteria for monoclonal B-cell lymphocytosis. Br J Haematol, 2005. 130(3): p. 325-32.
40. Rai, K.R., et al., Clinical staging of chronic lymphocytic leukemia. Blood, 1975. 46(2): p. 219-34.
41. Binet, J.L., et al., A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer, 1981. 48(1): p. 198-206.
42. Rai, K.R., A critical analysis of staging in CLL. In: Gale RP, Rai KR, editors. Chronic Lymphocytic Leukemia: Recent Progress and Future Directions. New York, NY: Liss; 1987. p. 253-264. 1987.
43. Dighiero, G. and J.L. Binet, When and how to treat chronic lymphocytic leukemia. N Engl J Med, 2000. 343(24): p. 1799-801.
44. Montserrat, E., Classical and new prognostic factors in chronic lymphocytic leukemia: where to now? Hematol J, 2002. 3(1): p. 7-9.
45. Molica, S. and A. Alberti, Prognostic value of the lymphocyte doubling time in chronic lymphocytic leukemia. Cancer, 1987. 60(11): p. 2712-6.
46. Vinolas, N., et al., Lymphocyte doubling time in chronic lymphocytic leukemia: an update of its prognostic significance. Blood Cells, 1987. 12(2): p. 457-70.

47. Molica, S., et al., Timing of diagnosis and lymphocyte accumulation patterns in chronic lymphocytic leukemia: analysis of their clinical significance. Eur J Haematol, 1990. 44(5): p. 277-81.

48. Montserrat, E., et al., Bone marrow assessment in B-cell chronic lymphocytic leukaemia: aspirate or biopsy? A comparative study in 258 patients. Br J Haematol, 1996. 93(1): p. 111-6.

49. Gentile, M., et al., Predictive value of beta2-microglobulin (beta2-m) levels in chronic lymphocytic leukemia since Binet A stages. Haematologica, 2009. 94(6): p. 887-8.

50. Hallek, M., et al., Serum beta(2)-microglobulin and serum thymidine kinase are independent predictors of progression-free survival in chronic lymphocytic leukemia and immunocytoma. Leuk Lymphoma, 1996. 22(5-6): p. 439-47.

51. Delgado, J., et al., Beta2-microglobulin is a better predictor of treatment-free survival in patients with chronic lymphocytic leukaemia if adjusted according to glomerular filtration rate. Br J Haematol, 2009. 145(6): p. 801-5.

52. Gronowitz, J.S., et al., The use of serum deoxythymidine kinase as a prognostic marker, and in the monitoring of patients with non-Hodgkin's lymphoma. Br J Cancer, 1983. 47(4): p. 487-95.

53. Kallander, C.F., et al., Serum deoxythymidine kinase gives prognostic information in chronic lymphocytic leukemia. Cancer, 1984. 54(11): p. 2450-5.

54. Magnac, C., et al., Predictive value of serum thymidine kinase level for Ig-V mutational status in B-CLL. Leukemia, 2003. 17(1): p. 133-7.

55. Molica, S., et al., Cellular expression and serum circulating levels of CD23 in B-cell chronic lymphocytic leukemia. Implications for prognosis. Haematologica, 1996. 81(5): p. 428-33.

56. Sarfati, M., et al., Prognostic importance of serum soluble CD23 level in chronic lymphocytic leukemia. Blood, 1996. 88(11): p. 4259-64.

57. Bergmann, M.A., Prospective evaluation of prognostic parameters in early stage chronic lymphocytic leukemia (CLL): results of the CLL1-protocol of the German CLL Study Group (GCLLSG) [abstract]. Blood 2007.

58. Lee, J.S., et al., Prognosis of chronic lymphocytic leukemia: a multivariate regression analysis of 325 untreated patients. Blood, 1987. 69(3): p. 929-36.

59. Van Bockstaele, F., B. Verhasselt, and J. Philippe, Prognostic markers in chronic lymphocytic leukemia: a comprehensive review. Blood Rev, 2009. 23(1): p. 25-47.

60. Damle, R.N., et al., Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood, 1999. 94(6): p. 1840-7.

61. Hamblin, T.J., et al., Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood, 1999. 94(6): p. 1848-54.

62. Deaglio, S., et al., CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. Blood, 2003. 102(6): p. 2146-55.

63. Ibrahim, S., et al., CD38 expression as an important prognostic factor in B-cell chronic lymphocytic leukemia. Blood, 2001. 98(1): p. 181-6.

64. Guarini, A., et al., Chronic lymphocytic leukemia patients with highly stable and indolent disease show distinctive phenotypic and genotypic features. Blood, 2003. 102(3): p. 1035-41.

65. Hamblin, T.J., et al., CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. Blood, 2002. 99(3): p. 1023-9.

66. Ghia, P., et al., CD38 modifications in chronic lymphocytic leukemia: are they relevant? Leukemia, 2004. 18(10): p. 1733-5.

67. Chen, L., et al., Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. Blood, 2002. 100(13): p. 4609-14.

68. Castro, J.E., et al., ZAP-70 is a novel conditional heat shock protein 90 (Hsp90) client: inhibition of Hsp90 leads to ZAP-70 degradation, apoptosis, and impaired signaling in chronic lymphocytic leukemia. Blood, 2005. 106(7): p. 2506-12.

69. Wiestner, A., et al., ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood, 2003. 101(12): p. 4944-51.

70. Durig, J., et al., ZAP-70 expression is a prognostic factor in chronic lymphocytic leukemia. Leukemia, 2003. 17(12): p. 2426-34.

71. Rassenti, L.Z., et al., ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. N Engl J Med, 2004. 351(9): p. 893-901.

72. Bosch, F., et al., Clinical implications of ZAP-70 expressionin chronic lymphocytic leukemia. Cytometry B Clin Cytom, 2006. 70(4): p. 214-7.

73. Rassenti, L.Z., et al., Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia. Blood, 2008. 112(5): p. 1923-30.

74. Krober, A., et al., V(H) mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. Blood, 2002. 100(4): p. 1410-6.

75. Dohner, H., et al., Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med, 2000. 343(26): p. 1910-6.

76. Oscier, D.G., et al., Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. Blood, 2002. 100(4): p. 1177-84.

77. Dewald, G.W., et al., Chromosome anomalies detected by interphase fluorescence in situ hybridization: correlation with significant biological features of B-cell chronic lymphocytic leukaemia. Br J Haematol, 2003. 121(2): p. 287-95.

78. Dohner, H., et al., 11q deletions identify a new subset of B-cell chronic lymphocytic leukemia characterized by extensive nodal involvement and inferior prognosis. Blood, 1997. 89(7): p. 2516-22.

79. Caligaris-Cappio, F. and P. Ghia, The normal counterpart to the chronic lymphocytic leukemia B cell. Best Pract Res Clin Haematol, 2007. 20(3): p. 385-97.

80. Liu, Y.J., et al., Mechanism of antigen-driven selection in germinal centres. Nature, 1989. 342(6252): p. 929-31.

81. Klein, U. and R. Dalla-Favera, Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol, 2008. 8(1): p. 22-33.

82. Wabl, M., M. Cascalho, and C. Steinberg, Hypermutation in antibody affinity maturation. Curr Opin Immunol, 1999. 11(2): p. 186-9.

83. Vuillier, F., et al., Lower levels of surface B-cell-receptor expression in chronic lymphocytic leukemia are associated with glycosylation and folding defects of the mu and CD79a chains. Blood, 2005. 105(7): p. 2933-40.

84. Thompson, A.A., et al., Aberrations of the B-cell receptor B29 (CD79b) gene in chronic lymphocytic leukemia. Blood, 1997. 90(4): p. 1387-94.

85. Alfarano, A., et al., An alternatively spliced form of CD79b gene may account for altered B-cell receptor expression in B-chronic lymphocytic leukemia. Blood, 1999. 93(7): p. 2327-35.

86. Payelle-Brogard, B., et al., Analysis of the B-cell receptor B29 (CD79b) gene in familial chronic lymphocytic leukemia. Blood, 1999. 94(10): p. 3516-22.

87. Schroeder, H.W., Jr. and G. Dighiero, The pathogenesis of chronic lymphocytic leukemia: analysis of the antibody repertoire. Immunol Today, 1994. 15(6): p. 288-94.

88. Fais, F., et al., Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest, 1998. 102(8): p. 1515-25.

89. Damle, R.N., et al., Telomere length and telomerase activity delineate distinctive replicative features of the B-CLL subgroups defined by immunoglobulin V gene mutations. Blood, 2004. 103(2): p. 375-82.

90. Damle, R.N., et al., B-cell chronic lymphocytic leukemia cells express a surface membrane phenotype of activated, antigen-experienced B lymphocytes. Blood, 2002. 99(11): p. 4087-93.

91. Oscier, D.G., et al., Differential rates of somatic hypermutation in V(H) genes among subsets of chronic lymphocytic leukemia defined by chromosomal abnormalities. Blood, 1997. 89(11): p. 4153-60.
92. Oppezzo, P., et al., Chronic lymphocytic leukemia B cells expressing AID display dissociation between class switch recombination and somatic hypermutation. Blood, 2003. 101(10): p. 4029-32.
93. Oppezzo, P., et al., Different isoforms of BSAP regulate expression of AID in normal and chronic lymphocytic leukemia B cells. Blood, 2005. 105(6): p. 2495-503.
94. McCarthy, H., et al., High expression of activation-induced cytidine deaminase (AID) and splice variants is a distinctive feature of poor-prognosis chronic lymphocytic leukemia. Blood, 2003. 101(12): p. 4903-8.
95. Messmer, B.T., et al., The pattern and distribution of immunoglobulin VH gene mutations in chronic lymphocytic leukemia B cells are consistent with the canonical somatic hypermutation process. Blood, 2004. 103(9): p. 3490-5.
96. Hadzidimitriou, A., et al., Evidence for the significant role of immunoglobulin light chains in antigen recognition and selection in chronic lymphocytic leukemia. Blood, 2009. 113(2): p. 403-11.
97. Widhopf, G.F., 2nd, et al., Nonstochastic pairing of immunoglobulin heavy and light chains expressed by chronic lymphocytic leukemia B cells is predicated on the heavy chain CDR3. Blood, 2008. 111(6): p. 3137-44.
98. Ghia, P., et al., Geographic patterns and pathogenetic implications of IGHV gene usage in chronic lymphocytic leukemia: the lesson of the IGHV3-21 gene. Blood, 2005. 105(4): p. 1678-85.
99. Stamatopoulos, K., et al., Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: Pathogenetic implications and clinical correlations. Blood, 2007. 109(1): p. 259-70.
100. Messmer, B.T., et al., Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J Exp Med, 2004. 200(4): p. 519-25.
101. Widhopf, G.F., 2nd, et al., Chronic lymphocytic leukemia B cells of more than 1% of patients express virtually identical immunoglobulins. Blood, 2004. 104(8): p. 2499-504.
102. Murray, F., et al., Stereotyped patterns of somatic hypermutation in subsets of patients with chronic lymphocytic leukemia: implications for the role of antigen selection in leukemogenesis. Blood, 2008. 111(3): p. 1524-33.
103. Tobin, G., et al., Somatically mutated Ig V(H)3-21 genes characterize a new subset of chronic lymphocytic leukemia. Blood, 2002. 99(6): p. 2262-4.
104. Chu, C.C., et al., Chronic lymphocytic leukemia antibodies with a common stereotypic rearrangement recognize nonmuscle myosin heavy chain IIA. Blood, 2008. 112(13): p. 5122-9.
105. Sutton, L.A., et al., Extensive intraclonal diversification in a subgroup of chronic lymphocytic leukemia patients with stereotyped IGHV4-34 receptors: implications for ongoing interactions with antigen. Blood, 2009. 114(20): p. 4460-8.
106. Kostareli, E., et al., Molecular evidence for EBV and CMV persistence in a subset of patients with chronic lymphocytic leukemia expressing stereotyped IGHV4-34 B-cell receptors. Leukemia, 2009. 23(5): p. 919-24.
107. Lanemo Myhrinder, A., et al., A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. Blood, 2008. 111(7): p. 3838-48.
108. Weill, J.C., S. Weller, and C.A. Reynaud, Human marginal zone B cells. Annu Rev Immunol, 2009. 27: p. 267-85.
109. Calin, G.A., et al., Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A, 2002. 99(24): p. 15524-9.
110. Cimmino, A., et al., miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A, 2005. 102(39): p. 13944-9.

111. Stankovic, T., et al., Inactivation of ataxia telangiectasia mutated gene in B-cell chronic lymphocytic leukaemia. Lancet, 1999. 353(9146): p. 26-9.

112. Schaffner, C., et al., Somatic ATM mutations indicate a pathogenic role of ATM in B-cell chronic lymphocytic leukemia. Blood, 1999. 94(2): p. 748-53.

113. Raval, A., J.C. Byrd, and C. Plass, Epigenetics in chronic lymphocytic leukemia. Semin Oncol, 2006. 33(2): p. 157-66.

114. Plass, C., et al., Molecular profiling of chronic lymphocytic leukaemia: genetics meets epigenetics to identify predisposing genes. Br J Haematol, 2007. 139(5): p. 744-52.

115. Hanada, M., et al., bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. Blood, 1993. 82(6): p. 1820-8.

116. Yuille, M.R., et al., TCL1 is activated by chromosomal rearrangement or by hypomethylation. Genes Chromosomes Cancer, 2001. 30(4): p. 336-41.

117. Rush, L.J., et al., Epigenetic profiling in chronic lymphocytic leukemia reveals novel methylation targets. Cancer Res, 2004. 64(7): p. 2424-33.

118. Raval, A., et al., Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. Cell, 2007. 129(5): p. 879-90.

119. Corcoran, M., et al., ZAP-70 methylation status is associated with ZAP-70 expression status in chronic lymphocytic leukemia. Haematologica, 2005. 90(8): p. 1078-88.

120. Byrd, J.C., et al., Depsipeptide (FR901228): a novel therapeutic agent with selective, in vitro activity against human B-cell chronic lymphocytic leukemia cells. Blood, 1999. 94(4): p. 1401-8.

121. Collins, R.J., et al., Spontaneous programmed death (apoptosis) of B-chronic lymphocytic leukaemia cells following their culture in vitro. Br J Haematol, 1989. 71(3): p. 343-50.

122. Burger, J.A. and T.J. Kipps, Chemokine receptors and stromal cells in the homing and homeostasis of chronic lymphocytic leukemia B cells. Leuk Lymphoma, 2002. 43(3): p. 461-6.

123. Caligaris-Cappio, F., Role of the microenvironment in chronic lymphocytic leukaemia. Br J Haematol, 2003. 123(3): p. 380-8.

124. Burger, J.A., et al., Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood, 2000. 96(8): p. 2655-63.

125. Lagneaux, L., et al., Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. Blood, 1998. 91(7): p. 2387-96.

126. Panayiotidis, P., et al., Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro. Br J Haematol, 1996. 92(1): p. 97-103.

127. Pedersen, I.M., et al., Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of Mcl-1. Blood, 2002. 100(5): p. 1795-801.

128. Munk Pedersen, I. and J. Reed, Microenvironmental interactions and survival of CLL B-cells. Leuk Lymphoma, 2004. 45(12): p. 2365-72.

129. Bertilaccio, M.T., et al., An overview of chronic lymphocytic leukaemia biology. Best Pract Res Clin Haematol, 2010. 23(1): p. 21-32.

130. Schmid, C. and P.G. Isaacson, Proliferation centres in B-cell malignant lymphoma, lymphocytic (B-CLL): an immunophenotypic study. Histopathology, 1994. 24(5): p. 445-51.

131. Soma, L.A., F.E. Craig, and S.H. Swerdlow, The proliferation center microenvironment and prognostic markers in chronic lymphocytic leukemia/small lymphocytic lymphoma. Hum Pathol, 2006. 37(2): p. 152-9.

132. Ghia, P., et al., Differential effects on CLL cell survival exerted by different microenvironmental elements. Curr Top Microbiol Immunol, 2005. 294: p. 135-45.

133. Nishio, M., et al., Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha. Blood, 2005. 106(3): p. 1012-20.

134. Granziero, L., et al., Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia. Blood, 2001. 97(9): p. 2777-83.

135. Francis, D.A., et al., Induction of the transcription factors NF-kappa B, AP-1 and NF-AT during B cell stimulation through the CD40 receptor. Int Immunol, 1995. 7(2): p. 151-61.

136. Chemotherapeutic options in chronic lymphocytic leukemia: a meta-analysis of the randomized trials. CLL Trialists' Collaborative Group. J Natl Cancer Inst, 1999. 91(10): p. 861-8.

137. Knauf, W.U., et al., Phase III randomized study of bendamustine compared with chlorambucil in previously untreated patients with chronic lymphocytic leukemia. J Clin Oncol, 2009. 27(26): p. 4378-84.

138. Johnson, S., et al., Multicentre prospective randomised trial of fludarabine versus cyclophosphamide, doxorubicin, and prednisone (CAP) for treatment of advanced-stage chronic lymphocytic leukaemia. The French Cooperative Group on CLL. Lancet, 1996. 347(9013): p. 1432-8.

139. Leporrier, M., et al., Randomized comparison of fludarabine, CAP, and ChOP in 938 previously untreated stage B and C chronic lymphocytic leukemia patients. Blood, 2001. 98(8): p. 2319-25.

140. Yamauchi, T., et al., DNA repair initiated in chronic lymphocytic leukemia lymphocytes by 4-hydroperoxycyclophosphamide is inhibited by fludarabine and clofarabine. Clin Cancer Res, 2001. 7(11): p. 3580-9.

141. Keating, M.J., et al., Long-term follow-up of patients with chronic lymphocytic leukemia (CLL) receiving fludarabine regimens as initial therapy. Blood, 1998. 92(4): p. 1165-71.

142. Rai, K.R., et al., Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia. N Engl J Med, 2000. 343(24): p. 1750-7.

143. Hallek, M., State-of-the-art treatment of chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program, 2009: p. 440-9.

144. Catovsky, D., et al., Assessment of fludarabine plus cyclophosphamide for patients with chronic lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled trial. Lancet, 2007. 370(9583): p. 230-9.

145. Flinn, I.W., et al., Phase III trial of fludarabine plus cyclophosphamide compared with fludarabine for patients with previously untreated chronic lymphocytic leukemia: US Intergroup Trial E2997. J Clin Oncol, 2007. 25(7): p. 793-8.

146. Eichhorst, B.F., et al., Fludarabine plus cyclophosphamide versus fludarabine alone in first-line therapy of younger patients with chronic lymphocytic leukemia. Blood, 2006. 107(3): p. 885-91.

147. Treumann, A., et al., Primary structure of CD52. J Biol Chem, 1995. 270(11): p. 6088-99.

148. Villamor, N., E. Montserrat, and D. Colomer, Mechanism of action and resistance to monoclonal antibody therapy. Semin Oncol, 2003. 30(4): p. 424-33.

149. Karlsson, C., et al., Phase II study of subcutaneous alemtuzumab without dose escalation in patients with advanced-stage, relapsed chronic lymphocytic leukaemia. Br J Haematol, 2009. 144(1): p. 78-85.

150. Bowen, A.L., et al., Subcutaneous CAMPATH-1H in fludarabine-resistant/relapsed chronic lymphocytic and B-prolymphocytic leukaemia. Br J Haematol, 1997. 96(3): p. 617-9.

151. Keating, M.J., et al., Therapeutic role of alemtuzumab (Campath-1H) in patients who have failed fludarabine: results of a large international study. Blood, 2002. 99(10): p. 3554-61.

152. Stilgenbauer, S. and H. Dohner, Campath-1H-induced complete remission of chronic lymphocytic leukemia despite p53 gene mutation and resistance to chemotherapy. N Engl J Med, 2002. 347(6): p. 452-3.

153. Osterborg, A., et al., Phase II multicenter study of human CD52 antibody in previously treated chronic lymphocytic leukemia. European Study Group of CAMPATH-1H Treatment in Chronic Lymphocytic Leukemia. J Clin Oncol, 1997. 15(4): p. 1567-74.

154. Stilgenbauer, S., et al., Subcutaneous alemtuzumab in fludarabine-refractory chronic lymphocytic leukemia: clinical results and prognostic marker analyses from the CLL2H study of the German Chronic Lymphocytic Leukemia Study Group. J Clin Oncol, 2009. 27(24): p. 3994-4001.

155. Lundin, J., et al., Phase II trial of subcutaneous anti-CD52 monoclonal antibody alemtuzumab (Campath-1H) as first-line treatment for patients with B-cell chronic lymphocytic leukemia (B-CLL). Blood, 2002. 100(3): p. 768-73.

156. Hillmen, P., et al., Alemtuzumab compared with chlorambucil as first-line therapy for chronic lymphocytic leukemia. J Clin Oncol, 2007. 25(35): p. 5616-23.

157. Hainsworth, J.D., et al., Single-agent rituximab as first-line and maintenance treatment for patients with chronic lymphocytic leukemia or small lymphocytic lymphoma: a phase II trial of the Minnie Pearl Cancer Research Network. J Clin Oncol, 2003. 21(9): p. 1746-51.

158. Itala, M., et al., Standard-dose anti-CD20 antibody rituximab has efficacy in chronic lymphocytic leukaemia: results from a Nordic multicentre study. Eur J Haematol, 2002. 69(3): p. 129-34.

159. Huhn, D., et al., Rituximab therapy of patients with B-cell chronic lymphocytic leukemia. Blood, 2001. 98(5): p. 1326-31.

160. Teeling, J.L., et al., Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. Blood, 2004. 104(6): p. 1793-800.

161. Pawluczkowycz, A.W., et al., Binding of submaximal C1q promotes complement-dependent cytotoxicity (CDC) of B cells opsonized with anti-CD20 mAbs ofatumumab (OFA) or rituximab (RTX): considerably higher levels of CDC are induced by OFA than by RTX. J Immunol, 2009. 183(1): p. 749-58.

162. Wierda, W.G., et al., Ofatumumab as single-agent CD20 immunotherapy in fludarabine-refractory chronic lymphocytic leukemia. J Clin Oncol, 2010. 28(10): p. 1749-55.

163. Alas, S., B. Bonavida, and C. Emmanouilides, Potentiation of fludarabine cytotoxicity on non-Hodgkin's lymphoma by pentoxifylline and rituximab. Anticancer Res, 2000. 20(5A): p. 2961-6.

164. Demidem, A., et al., Chimeric anti-CD20 (IDEC-C2B8) monoclonal antibody sensitizes a B cell lymphoma cell line to cell killing by cytotoxic drugs. Cancer Biother Radiopharm, 1997. 12(3): p. 177-86.

165. Di Gaetano, N., et al., Synergism between fludarabine and rituximab revealed in a follicular lymphoma cell line resistant to the cytotoxic activity of either drug alone. Br J Haematol, 2001. 114(4): p. 800-9.

166. Schulz, H., et al., Phase 2 study of a combined immunochemotherapy using rituximab and fludarabine in patients with chronic lymphocytic leukemia. Blood, 2002. 100(9): p. 3115-20.

167. Del Poeta, G., et al., The addition of rituximab to fludarabine improves clinical outcome in untreated patients with ZAP-70-negative chronic lymphocytic leukemia. Cancer, 2005. 104(12): p. 2743-52.

168. Byrd, J.C., et al., Randomized phase 2 study of fludarabine with concurrent versus sequential treatment with rituximab in symptomatic, untreated patients with B-cell chronic lymphocytic leukemia: results from Cancer and Leukemia Group B 9712 (CALGB 9712). Blood, 2003. 101(1): p. 6-14.

169. Byrd, J.C., et al., Addition of rituximab to fludarabine may prolong progression-free survival and overall survival in patients with previously untreated chronic lymphocytic leukemia: an updated retrospective comparative analysis of CALGB 9712 and CALGB 9011. Blood, 2005. 105(1): p. 49-53.

170. Keating, M.J., et al., Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia. J Clin Oncol, 2005. 23(18): p. 4079-88.

171. Hallek, M., et al., Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. Lancet, 2010. 376(9747): p. 1164-74.

172. Lepretre, S., et al., Immunochemotherapy with fludarabine (F), cyclophosphamide (C), and rituximab (R) (FCR) versus fludarabine (F), cyclophosphamide (C), and mabcampath (CAM) (FCCAM) in previously untreated patients (pts) with advanced B-chronic lymphocytic leukemia (B-CLL): experience on safety and efficacy with a randomised multicenter phase III trial of the French cooperative group in CLL and WM (FCGCLL/MW) and the "Groupe ouest-est d´etudes

des leucemés aigues et autres maladies du sang" (GOELAMS): CLL2007FMP (for fit medically patients). Haematologica, October 2009. 94(supplement 3):abs 75.

173. Sutton, L., et al., Autologous hematopoietic stem cell transplantation as salvage treatment for advanced B cell chronic lymphocytic leukemia. Leukemia, 1998. 12(11): p. 1699-707.
174. Pavletic, Z.S., et al., High incidence of relapse after autologous stem-cell transplantation for B-cell chronic lymphocytic leukemia or small lymphocytic lymphoma. Ann Oncol, 1998. 9(9): p. 1023-6.
175. Montserrat, E., Role of auto- and allotransplantation in B-cell chronic lymphocytic leukemia. Hematol Oncol Clin North Am, 2004. 18(4): p. 915-26, x.
176. Dreger, P., et al., Indications for allogeneic stem cell transplantation in chronic lymphocytic leukemia: the EBMT transplant consensus. Leukemia, 2007. 21(1): p. 12-7.
177. Gribben, J.G. and S. O'Brien, Update on therapy of chronic lymphocytic leukemia. J Clin Oncol, 2011. 29(5): p. 544-50.
178. Riches, J.C., A.G. Ramsay, and J.G. Gribben, Chronic lymphocytic leukemia: an update on biology and treatment. Curr Oncol Rep, 2011. 13(5): p. 379-85.
179. Garcia-Escobar, I., et al., Therapeutic management of chronic lymphocytic leukaemia State of the art and future perspectives. Crit Rev Oncol Hematol, 2011. 80(1): p. 100-113.
180. Chanan-Khan, A., et al., Clinical efficacy of lenalidomide in patients with relapsed or refractory chronic lymphocytic leukemia: results of a phase II study. J Clin Oncol, 2006. 24(34): p. 5343-9.
181. Ferrajoli, A., et al., Lenalidomide induces complete and partial remissions in patients with relapsed and refractory chronic lymphocytic leukemia. Blood, 2008. 111(11): p. 5291-7.
182. Byrd, J.C., et al., Phase 1/2 study of lumiliximab combined with fludarabine, cyclophosphamide, and rituximab in patients with relapsed or refractory chronic lymphocytic leukemia. Blood, 2010. 115(3): p. 489-95.
183. Abou-Nassar, K. and J.R. Brown, Novel agents for the treatment of chronic lymphocytic leukemia. Clin Adv Hematol Oncol, 2010. 8(12): p. 886-95.
184. Morschhauser, F., et al., Phase I study of RO5072759 (GA101) in relapsed/refractory chronic lymphocytic leukemia. Blood (ASH Annual Meeting Abstracts), 2009. 114. Abstract 884.
185. Bologna, L., et al., Mechanism of action of type II, glycoengineered, anti-CD20 monoclonal antibody GA101 in B-chronic lymphocytic leukemia whole blood assays in comparison with rituximab and alemtuzumab. J Immunol, 2011. 186(6): p. 3762-9.
186. Hallek, M. and N. Pflug, State of the art treatment of chronic lymphocytic leukaemia. Blood Rev, 2011. 25(1): p. 1-9.
187. Alduaij, W., et al., Novel type II anti-CD20 monoclonal antibody (GA101) evokes homotypic adhesion and actin-dependent, lysosome-mediated cell death in B-cell malignancies. Blood, 2011. 117(17): p. 4519-29.
188. Goede, V. and M. Hallek, Optimal pharmacotherapeutic management of chronic lymphocytic leukaemia: considerations in the elderly. Drugs Aging, 2011. 28(3): p. 163-76.
189. Ahmadi, T., et al., Chronic lymphocytic leukemia: new concepts and emerging therapies. Curr Treat Options Oncol, 2009. 10(1-2): p. 16-32.
190. Lin, T.S., et al., Phase II study of flavopiridol in relapsed chronic lymphocytic leukemia demonstrating high response rates in genetically high-risk disease. J Clin Oncol, 2009. 27(35): p. 6012-8.
191. Coutre, S.E., et al., Phase I study of CAL-101, an isoform-selective inhibitor of phosphatidylinositol 3‑ kinase P110d, in patients with previously treated chronic lymphocytic leukemia. J Clin Oncol, 2011. 29(suppl; abstr 6631).
192. Sadelain, M., R. Brentjens, and I. Riviere, The promise and potential pitfalls of chimeric antigen receptors. Curr Opin Immunol, 2009. 21(2): p. 215-23.
193. Jena, B., G. Dotti, and L.J. Cooper, Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. Blood, 2010. 116(7): p. 1035-44.

194. Irving, B.A. and A. Weiss, The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. Cell, 1991. 64(5): p. 891-901.

195. Carpenito, C., et al., Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. Proc Natl Acad Sci U S A, 2009. 106(9): p. 3360-5.

196. Kalos, M., et al., T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced Leukemia. Sci Transl Med, 2011. 3(95): p. 95ra73.

197. Porter, D.L., et al., Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med, 2011. 365(8): p. 725-33.

198. Jaglowski, S.M., et al., The clinical application of monoclonal antibodies in chronic lymphocytic leukemia. Blood, 2010. 116(19): p. 3705-14.

199. Gschwind, A., O.M. Fischer, and A. Ullrich, The discovery of receptor tyrosine kinases: targets for cancer therapy. Nat Rev Cancer, 2004. 4(5): p. 361-70.

200. Blume-Jensen, P. and T. Hunter, Oncogenic kinase signalling. Nature, 2001. 411(6835): p. 355-65.

201. Ullrich, A. and J. Schlessinger, Signal transduction by receptors with tyrosine kinase activity. Cell, 1990. 61(2): p. 203-12.

202. Robinson, D.R., Y.M. Wu, and S.F. Lin, The protein tyrosine kinase family of the human genome. Oncogene, 2000. 19(49): p. 5548-57.

203. Schlessinger, J., Cell signaling by receptor tyrosine kinases. Cell, 2000. 103(2): p. 211-25.

204. Lemmon, M.A. and J. Schlessinger, Cell signaling by receptor tyrosine kinases. Cell, 2010. 141(7): p. 1117-34.

205. Haglund, K., T.E. Rusten, and H. Stenmark, Aberrant receptor signaling and trafficking as mechanisms in oncogenesis. Crit Rev Oncog, 2007. 13(1): p. 39-74.

206. Abella, J.V. and M. Park, Breakdown of endocytosis in the oncogenic activation of receptor tyrosine kinases. Am J Physiol Endocrinol Metab, 2009. 296(5): p. E973-84.

207. Witsch, E., M. Sela, and Y. Yarden, Roles for growth factors in cancer progression. Physiology (Bethesda), 2010. 25(2): p. 85-101.

208. Bennasroune, A., et al., Tyrosine kinase receptors as attractive targets of cancer therapy. Crit Rev Oncol Hematol, 2004. 50(1): p. 23-38.

209. Hubbard, S.R. and J.H. Till, Protein tyrosine kinase structure and function. Annu Rev Biochem, 2000. 69: p. 373-98.

210. Watson, M.E., Compilation of published signal sequences. Nucleic Acids Res, 1984. 12(13): p. 5145-64.

211. Siegel, G.J., et al., Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 2006.

212. Chinkers, M. and J.S. Brugge, Characterization of structural domains of the human epidermal growth factor receptor obtained by partial proteolysis. J Biol Chem, 1984. 259(18): p. 11534-42.

213. Mayes, E.L. and M.D. Waterfield, Biosynthesis of the epidermal growth factor receptor in A431 cells. EMBO J, 1984. 3(3): p. 531-7.

214. Blobel, G., Intracellular protein topogenesis. Proc Natl Acad Sci U S A, 1980. 77(3): p. 1496-500.

215. Yarden, Y. and A. Ullrich, Growth factor receptor tyrosine kinases. Annu Rev Biochem, 1988. 57: p. 443-78.

216. Hubbard, S.R., Theme and variations: juxtamembrane regulation of receptor protein kinases. Mol Cell, 2001. 8(3): p. 481-2.

217. Hunter, T. and J.A. Cooper, Protein-tyrosine kinases. Annu Rev Biochem, 1985. 54: p. 897-930.

218. Hubbard, S.R., Structural analysis of receptor tyrosine kinases. Prog Biophys Mol Biol, 1999. 71(3-4): p. 343-58.

219. Krause, D.S. and R.A. Van Etten, Tyrosine kinases as targets for cancer therapy. N Engl J Med, 2005. 353(2): p. 172-87.

220. Griffith, J., et al., The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. Mol Cell, 2004. 13(2): p. 169-78.

221. Heldin, C.H., Dimerization of cell surface receptors in signal transduction. Cell, 1995. 80(2): p. 213-23.

222. Weiss, A. and J. Schlessinger, Switching signals on or off by receptor dimerization. Cell, 1998. 94(3): p. 277-80.

223. Olayioye, M.A., et al., The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J, 2000. 19(13): p. 3159-67.

224. Yarden, Y. and M.X. Sliwkowski, Untangling the ErbB signalling network. Nat Rev Mol Cell Biol, 2001. 2(2): p. 127-37.

225. Wiesmann, C., et al., Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. Nature, 1999. 401(6749): p. 184-8.

226. Sasaki, T., et al., Structural basis for Gas6-Axl signalling. EMBO J, 2006. 25(1): p. 80-7.

227. Himanen, J.P. and D.B. Nikolov, Eph signaling: a structural view. Trends Neurosci, 2003. 26(1): p. 46-51.

228. Garrett, T.P., et al., Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. Cell, 2002. 110(6): p. 763-73.

229. Ogiso, H., et al., Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. Cell, 2002. 110(6): p. 775-87.

230. Burgess, A.W., et al., An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. Mol Cell, 2003. 12(3): p. 541-52.

231. Bouyain, S., et al., The extracellular region of ErbB4 adopts a tethered conformation in the absence of ligand. Proc Natl Acad Sci U S A, 2005. 102(42): p. 15024-9.

232. Cho, H.S. and D.J. Leahy, Structure of the extracellular region of HER3 reveals an interdomain tether. Science, 2002. 297(5585): p. 1330-3.

233. Ferguson, K.M., et al., EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Mol Cell, 2003. 11(2): p. 507-17.

234. Hynes, N.E. and H.A. Lane, ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer, 2005. 5(5): p. 341-54.

235. Sierke, S.L., et al., Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein. Biochem J, 1997. 322 (Pt 3): p. 757-63.

236. Baselga, J. and S.M. Swain, Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. Nat Rev Cancer, 2009. 9(7): p. 463-75.

237. Liu, H., et al., Structural basis for stem cell factor-KIT signaling and activation of class III receptor tyrosine kinases. EMBO J, 2007. 26(3): p. 891-901.

238. Yuzawa, S., et al., Structural basis for activation of the receptor tyrosine kinase KIT by stem cell factor. Cell, 2007. 130(2): p. 323-34.

239. Kuriyan, J. and D. Cowburn, Modular peptide recognition domains in eukaryotic signaling. Annu Rev Biophys Biomol Struct, 1997. 26: p. 259-88.

240. Hanks, S.K. and A.M. Quinn, Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods Enzymol, 1991. 200: p. 38-62.

241. Ellis, L., et al., Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell, 1986. 45(5): p. 721-32.

242. Mohammadi, M., et al., Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. Mol Cell Biol, 1996. 16(3): p. 977-89.

243. Kendall, R.L., et al., Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. J Biol Chem, 1999. 274(10): p. 6453-60.

244. Fantl, W.J., J.A. Escobedo, and L.T. Williams, Mutations of the platelet-derived growth factor receptor that cause a loss of ligand-induced conformational change, subtle changes in kinase activity, and impaired ability to stimulate DNA synthesis. Mol Cell Biol, 1989. 9(10): p. 4473-8.

245. Longati, P., et al., Tyrosines1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). Oncogene, 1994. 9(1): p. 49-57.

246. Mitra, G., Mutational analysis of conserved residues in the tyrosine kinase domain of the human trk oncogene. Oncogene, 1991. 6(12): p. 2237-41.

247. Gotoh, N., et al., A highly conserved tyrosine residue at codon 845 within the kinase domain is not required for the transforming activity of human epidermal growth factor receptor. Biochem Biophys Res Commun, 1992. 186(2): p. 768-74.

248. Hubbard, S.R., M. Mohammadi, and J. Schlessinger, Autoregulatory mechanisms in protein-tyrosine kinases. J Biol Chem, 1998. 273(20): p. 11987-90.

249. Hubbard, S.R., Juxtamembrane autoinhibition in receptor tyrosine kinases. Nat Rev Mol Cell Biol, 2004. 5(6): p. 464-71.

250. Bae, J.H., et al., The selectivity of receptor tyrosine kinase signaling is controlled by a secondary SH2 domain binding site. Cell, 2009. 138(3): p. 514-24.

251. Chen, H., et al., A molecular brake in the kinase hinge region regulates the activity of receptor tyrosine kinases. Mol Cell, 2007. 27(5): p. 717-30.

252. Till, J.H., et al., Crystal structure of the MuSK tyrosine kinase: insights into receptor autoregulation. Structure, 2002. 10(9): p. 1187-96.

253. Mol, C.D., et al., Structural basis for the autoinhibition and STI-571 inhibition of c-Kit tyrosine kinase. J Biol Chem, 2004. 279(30): p. 31655-63.

254. Wybenga-Groot, L.E., et al., Structural basis for autoinhibition of the Ephb2 receptor tyrosine kinase by the unphosphorylated juxtamembrane region. Cell, 2001. 106(6): p. 745-57.

255. Shewchuk, L.M., et al., Structure of the Tie2 RTK domain: self-inhibition by the nucleotide binding loop, activation loop, and C-terminal tail. Structure, 2000. 8(11): p. 1105-13.

256. Sorkin, A. and L.K. Goh, Endocytosis and intracellular trafficking of ErbBs. Exp Cell Res, 2009. 315(4): p. 683-96.

257. von Zastrow, M. and A. Sorkin, Signaling on the endocytic pathway. Curr Opin Cell Biol, 2007. 19(4): p. 436-45.

258. Zwang, Y. and Y. Yarden, Systems biology of growth factor-induced receptor endocytosis. Traffic, 2009. 10(4): p. 349-63.

259. Waterman, H. and Y. Yarden, Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases. FEBS Lett, 2001. 490(3): p. 142-52.

260. Shtiegman, K. and Y. Yarden, The role of ubiquitylation in signaling by growth factors: implications to cancer. Semin Cancer Biol, 2003. 13(1): p. 29-40.

261. Citri, A., et al., Drug-induced ubiquitylation and degradation of ErbB receptor tyrosine kinases: implications for cancer therapy. EMBO J, 2002. 21(10): p. 2407-17.

262. Hunter, T., The age of crosstalk: phosphorylation, ubiquitination, and beyond. Mol Cell, 2007. 28(5): p. 730-8.

263. Nakao, M., et al., Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia, 1996. 10(12): p. 1911-8.

264. Lynch, T.J., et al., Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med, 2004. 350(21): p. 2129-39.

265. Pao, W., et al., EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A, 2004. 101(36): p. 13306-11.

266. Sordella, R., et al., Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science, 2004. 305(5687): p. 1163-7.

267. Slamon, D.J., et al., Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science, 1989. 244(4905): p. 707-12.

268. Finn, R.S. and D.J. Slamon, Monoclonal antibody therapy for breast cancer: herceptin. Cancer Chemother Biol Response Modif, 2003. 21: p. 223-33.
269. Baskar, S., et al., Unique cell surface expression of receptor tyrosine kinase ROR1 in human B-cell chronic lymphocytic leukemia. Clin Cancer Res, 2008. 14(2): p. 396-404.
270. Daneshmanesh, A.H., et al., Ror1, a cell surface receptor tyrosine kinase is expressed in chronic lymphocytic leukemia and may serve as a putative target for therapy. Int J Cancer, 2008. 123(5): p. 1190-5.
271. Fukuda, T., et al., Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a. Proc Natl Acad Sci U S A, 2008. 105(8): p. 3047-52.
272. Baselga, J., Targeting tyrosine kinases in cancer: the second wave. Science, 2006. 312(5777): p. 1175-8.
273. Chen, J.S., K. Lan, and M.C. Hung, Strategies to target HER2/neu overexpression for cancer therapy. Drug Resist Updat, 2003. 6(3): p. 129-36.
274. Hudis, C.A., Trastuzumab--mechanism of action and use in clinical practice. N Engl J Med, 2007. 357(1): p. 39-51.
275. Slamon, D.J., et al., Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med, 2001. 344(11): p. 783-92.
276. Vogel, C.L., et al., Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol, 2002. 20(3): p. 719-26.
277. Weiner, L.M., R. Surana, and S. Wang, Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol, 2010. 10(5): p. 317-27.
278. Mackenzie, M., K. Spithoff, and D. Jonker, Systemic therapy for advanced gastric cancer: a clinical practice guideline. Curr Oncol, 2011. 18(4): p. e202-9.
279. Kim, R., Cetuximab and panitumumab: are they interchangeable? Lancet Oncol, 2009. 10(12): p. 1140-1.
280. Specenier, P. and J.B. Vermorken, Cetuximab in the treatment of squamous cell carcinoma of the head and neck. Expert Rev Anticancer Ther, 2011. 11(4): p. 511-24.
281. Sunada, H., et al., Monoclonal antibody against epidermal growth factor receptor is internalized without stimulating receptor phosphorylation. Proc Natl Acad Sci U S A, 1986. 83(11): p. 3825-9.
282. Li, S., et al., Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell, 2005. 7(4): p. 301-11.
283. Dempke, W.C. and V. Heinemann, Ras mutational status is a biomarker for resistance to EGFR inhibitors in colorectal carcinoma. Anticancer Res, 2010. 30(11): p. 4673-7.
284. Miller, K., et al., Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. N Engl J Med, 2007. 357(26): p. 2666-76.
285. Hurwitz, H., et al., Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med, 2004. 350(23): p. 2335-42.
286. Sandler, A., et al., Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med, 2006. 355(24): p. 2542-50.
287. Ellis, L.M. and D.J. Hicklin, VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer, 2008. 8(8): p. 579-91.
288. Zustovich, F., et al., Second-line therapy for refractory renal-cell carcinoma. Crit Rev Oncol Hematol, 2011.
289. Druker, B.J., STI571 (Gleevec) as a paradigm for cancer therapy. Trends Mol Med, 2002. 8(4 Suppl): p. S14-8.
290. Druker, B.J., Imatinib as a paradigm of targeted therapies. Adv Cancer Res, 2004. 91: p. 1-30.
291. Buchdunger, E., et al., Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. J Pharmacol Exp Ther, 2000. 295(1): p. 139-45.

292. Joensuu, H., et al., Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. N Engl J Med, 2001. 344(14): p. 1052-6.

293. Herbst, R.S., M. Fukuoka, and J. Baselga, Gefitinib--a novel targeted approach to treating cancer. Nat Rev Cancer, 2004. 4(12): p. 956-65.

294. Minna, J.D. and J. Dowell, Erlotinib hydrochloride. Nat Rev Drug Discov, 2005. Suppl: p. S14-5.

295. Kris, M.G., et al., Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA, 2003. 290(16): p. 2149-58.

296. Shepherd, F.A., et al., Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med, 2005. 353(2): p. 123-32.

297. Moore, M.J., et al., Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol, 2007. 25(15): p. 1960-6.

298. Matsuda, T., et al., Expression of the receptor tyrosine kinase genes, Ror1 and Ror2, during mouse development. Mech Dev, 2001. 105(1-2): p. 153-6.

299. Minami, Y., et al., Ror-family receptor tyrosine kinases in noncanonical Wnt signaling: their implications in developmental morphogenesis and human diseases. Dev Dyn, 2010. 239(1): p. 1-15.

300. Masiakowski, P. and R.D. Carroll, A novel family of cell surface receptors with tyrosine kinase-like domain. J Biol Chem, 1992. 267(36): p. 26181-90.

301. Oishi, I., et al., Spatio-temporally regulated expression of receptor tyrosine kinases, mRor1, mRor2, during mouse development: implications in development and function of the nervous system. Genes Cells, 1999. 4(1): p. 41-56.

302. Patthy, L., et al., Kringles: modules specialized for protein binding. Homology of the gelatin-binding region of fibronectin with the kringle structures of proteases. FEBS Lett, 1984. 171(1): p. 131-6.

303. Rehn, M., et al., The frizzled motif: in how many different protein families does it occur? Trends Biochem Sci, 1998. 23(11): p. 415-7.

304. Saldanha, J., J. Singh, and D. Mahadevan, Identification of a Frizzled-like cysteine rich domain in the extracellular region of developmental receptor tyrosine kinases. Protein Sci, 1998. 7(8): p. 1632-5.

305. Green, J.L., S.G. Kuntz, and P.W. Sternberg, Ror receptor tyrosine kinases: orphans no more. Trends Cell Biol, 2008. 18(11): p. 536-44.

306. Forrester, W.C., The Ror receptor tyrosine kinase family. Cell Mol Life Sci, 2002. 59(1): p. 83-96.

307. Wilson, C., D.C. Goberdhan, and H. Steller, Dror, a potential neurotrophic receptor gene, encodes a Drosophila homolog of the vertebrate Ror family of Trk-related receptor tyrosine kinases. Proc Natl Acad Sci U S A, 1993. 90(15): p. 7109-13.

308. Koga, M., et al., Control of DAF-7 TGF-(alpha) expression and neuronal process development by a receptor tyrosine kinase KIN-8 in Caenorhabditis elegans. Development, 1999. 126(23): p. 5387-98.

309. Forrester, W.C., et al., A C. elegans Ror receptor tyrosine kinase regulates cell motility and asymmetric cell division. Nature, 1999. 400(6747): p. 881-5.

310. McKay, S.E., et al., Aplysia ror forms clusters on the surface of identified neuroendocrine cells. Mol Cell Neurosci, 2001. 17(5): p. 821-41.

311. Katoh, M., Comparative genomics on ROR1 and ROR2 orthologs. Oncol Rep, 2005. 14(5): p. 1381-4.

312. Rodriguez-Niedenfuhr, M., F. Prols, and B. Christ, Expression and regulation of ROR-1 during early avian limb development. Anat Embryol (Berl), 2004. 207(6): p. 495-502.

313. Stricker, S., et al., Cloning and expression pattern of chicken Ror2 and functional characterization of truncating mutations in Brachydactyly type B and Robinow syndrome. Dev Dyn, 2006. 235(12): p. 3456-65.

314. Hikasa, H., et al., The Xenopus receptor tyrosine kinase Xror2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling. Development, 2002. 129(22): p. 5227-39.

315. Duffaud, G.D., et al., Structure and function of the signal peptide. Current Topics in Membranes and Transport, 1985. 24: p. 65–104.

316. Yoda, A., I. Oishi, and Y. Minami, Expression and function of the Ror-family receptor tyrosine kinases during development: lessons from genetic analyses of nematodes, mice, and humans. J Recept Signal Transduct Res, 2003. 23(1): p. 1-15.

317. Oishi, I., et al., The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. Genes Cells, 2003. 8(7): p. 645-54.

318. Mikels, A.J. and R. Nusse, Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. PLoS Biol, 2006. 4(4): p. e115.

319. Hanks, S.K., A.M. Quinn, and T. Hunter, The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science, 1988. 241(4861): p. 42-52.

320. Pawson, T., Protein modules and signalling networks. Nature, 1995. 373(6515): p. 573-80.

321. Hudecek, M., et al., The B-cell tumor-associated antigen ROR1 can be targeted with T cells modified to express a ROR1-specific chimeric antigen receptor. Blood, 2010. 116(22): p. 4532-41.

322. Al-Shawi, R., et al., Expression of the Ror1 and Ror2 receptor tyrosine kinase genes during mouse development. Dev Genes Evol, 2001. 211(4): p. 161-71.

323. Nomi, M., et al., Loss of mRor1 enhances the heart and skeletal abnormalities in mRor2-deficient mice: redundant and pleiotropic functions of mRor1 and mRor2 receptor tyrosine kinases. Mol Cell Biol, 2001. 21(24): p. 8329-35.

324. Paganoni, S. and A. Ferreira, Neurite extension in central neurons: a novel role for the receptor tyrosine kinases Ror1 and Ror2. J Cell Sci, 2005. 118(Pt 2): p. 433-46.

325. DeChiara, T.M., et al., Ror2, encoding a receptor-like tyrosine kinase, is required for cartilage and growth plate development. Nat Genet, 2000. 24(3): p. 271-4.

326. Takeuchi, S., et al., Mouse Ror2 receptor tyrosine kinase is required for the heart development and limb formation. Genes Cells, 2000. 5(1): p. 71-8.

327. Klein, U., et al., Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med, 2001. 194(11): p. 1625-38.

328. Shabani, M., et al., Overexpression of orphan receptor tyrosine kinase Ror1 as a putative tumor-associated antigen in Iranian patients with acute lymphoblastic leukemia. Tumour Biol, 2007. 28(6): p. 318-26.

329. Shabani, M., et al., Expression profile of orphan receptor tyrosine kinase (ROR1) and Wilms' tumor gene 1 (WT1) in different subsets of B-cell acute lymphoblastic leukemia. Leuk Lymphoma, 2008. 49(7): p. 1360-7.

330. Yang, J., et al., Therapeutic Potential and Challenges of Targeting Receptor Tyrosine Kinase ROR1 with Monoclonal Antibodies in B-Cell Malignancies. PLoS One, 2011. 6(6): p. e21018.

331. Barna, G., et al., ROR1 expression is not a unique marker of CLL. Hematol Oncol, 2011. 29(1): p. 17-21.

332. Uhrmacher, S., et al., Use of the receptor tyrosine kinase-like orphan receptor 1 (ROR1) as a diagnostic tool in chronic lymphocytic leukemia (CLL). Leuk Res, 2011.

333. Rabbani, H., et al., Expression of ROR1 in patients with renal cancer--a potential diagnostic marker. Iran Biomed J, 2010. 14(3): p. 77-82.

334. Gentile, A., et al., Ror1 is a pseudokinase that is crucial for Met-driven tumorigenesis. Cancer Res, 2011. 71(8): p. 3132-41.

335. van Bokhoven, H., et al., Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome. Nat Genet, 2000. 25(4): p. 423-6.

336. Afzal, A.R., et al., Recessive Robinow syndrome, allelic to dominant brachydactyly type B, is caused by mutation of ROR2. Nat Genet, 2000. 25(4): p. 419-22.

337. Oldridge, M., et al., Dominant mutations in ROR2, encoding an orphan receptor tyrosine kinase, cause brachydactyly type B. Nat Genet, 2000. 24(3): p. 275-8.

338. Schwabe, G.C., et al., Distinct mutations in the receptor tyrosine kinase gene ROR2 cause brachydactyly type B. Am J Hum Genet, 2000. 67(4): p. 822-31.

339. Robinow, M., F.N. Silverman, and H.D. Smith, A newly recognized dwarfing syndrome. Am J Dis Child, 1969. 117(6): p. 645-51.

340. Butler, M.G. and W.B. Wadlington, Robinow syndrome: report of two patients and review of literature. Clin Genet, 1987. 31(2): p. 77-85.

341. Soliman, A.T., et al., Recessive Robinow syndrome: with emphasis on endocrine functions. Metabolism, 1998. 47(11): p. 1337-43.

342. Teebi, A.S., Autosomal recessive Robinow syndrome. Am J Med Genet, 1990. 35(1): p. 64-8.

343. Houlston, R.S. and I.K. Temple, Characteristic facies in type B brachydactyly? Clin Dysmorphol, 1994. 3(3): p. 224-7.

344. Gong, Y., et al., Brachydactyly type B: clinical description, genetic mapping to chromosome 9q, and evidence for a shared ancestral mutation. Am J Hum Genet, 1999. 64(2): p. 570-7.

345. Kobayashi, M., et al., Ror2 expression in squamous cell carcinoma and epithelial dysplasia of the oral cavity. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2009. 107(3): p. 398-406.

346. O'Connell, M.P., et al., The orphan tyrosine kinase receptor, ROR2, mediates Wnt5A signaling in metastatic melanoma. Oncogene, 2010. 29(1): p. 34-44.

347. Morioka, K., et al., Orphan receptor tyrosine kinase ROR2 as a potential therapeutic target for osteosarcoma. Cancer Sci, 2009. 100(7): p. 1227-33.

348. Wright, T.M., et al., Ror2, a developmentally regulated kinase, promotes tumor growth potential in renal cell carcinoma. Oncogene, 2009. 28(27): p. 2513-23.

349. Yamamoto, H., et al., Wnt5a signaling is involved in the aggressiveness of prostate cancer and expression of metalloproteinase. Oncogene, 2010. 29(14): p. 2036-46.

350. Kubo, T., et al., Resequencing and copy number analysis of the human tyrosine kinase gene family in poorly differentiated gastric cancer. Carcinogenesis, 2009. 30(11): p. 1857-64.

351. Chen, H., et al., In vitro and in vivo production of vascular endothelial growth factor by chronic lymphocytic leukemia cells. Blood, 2000. 96(9): p. 3181-7.

352. Kay, N.E., et al., B-CLL cells are capable of synthesis and secretion of both pro- and anti-angiogenic molecules. Leukemia, 2002. 16(5): p. 911-9.

353. Ferrajoli, A., et al., High levels of vascular endothelial growth factor receptor-2 correlate with shortened survival in chronic lymphocytic leukemia. Clin Cancer Res, 2001. 7(4): p. 795-9.

354. Ghosh, A.K., et al., The novel receptor tyrosine kinase Axl is constitutively active in B-cell chronic lymphocytic leukemia and acts as a docking site of nonreceptor kinases: implications for therapy. Blood, 2011. 117(6): p. 1928-37.

355. Lee, Y.K., et al., VEGF receptor phosphorylation status and apoptosis is modulated by a green tea component, epigallocatechin-3-gallate (EGCG), in B-cell chronic lymphocytic leukemia. Blood, 2004. 104(3): p. 788-94.

356. Shanafelt, T., et al., Phase II trials of single-agent anti-VEGF therapy for patients with chronic lymphocytic leukemia. Leuk Lymphoma, 2010. 51(12): p. 2222-9.

357. Paesler, J., et al., The vascular endothelial growth factor receptor tyrosine kinase inhibitors vatalanib and pazopanib potently induce apoptosis in chronic lymphocytic leukemia cells in vitro and in vivo. Clin Cancer Res, 2010. 16(13): p. 3390-8.

358. Huber, S., et al., Sorafenib induces cell death in chronic lymphocytic leukemia by translational downregulation of Mcl-1. Leukemia, 2011. 25(5): p. 838-47.

359. Rosenwald, A., et al., Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med, 2001. 194(11): p. 1639-47.

360. Clevers, H., Wnt/beta-catenin signaling in development and disease. Cell, 2006. 127(3): p. 469-80.

361. Lu, D., et al., Activation of the Wnt signaling pathway in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A, 2004. 101(9): p. 3118-23.

362. McWhirter, J.R., et al., Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia. Proc Natl Acad Sci U S A, 1999. 96(20): p. 11464-9.

363. Derksen, P.W., et al., Illegitimate WNT signaling promotes proliferation of multiple myeloma cells. Proc Natl Acad Sci U S A, 2004. 101(16): p. 6122-7.

364. Lagneaux, L., et al., Adhesion to bone marrow stroma inhibits apoptosis of chronic lymphocytic leukemia cells. Leuk Lymphoma, 1999. 35(5-6): p. 445-53.

365. Rattis, F.M., C. Voermans, and T. Reya, Wnt signaling in the stem cell niche. Curr Opin Hematol, 2004. 11(2): p. 88-94.

366. Reya, T. and H. Clevers, Wnt signalling in stem cells and cancer. Nature, 2005. 434(7035): p. 843-50.

367. Schambony, A. and D. Wedlich, Wnt-5A/Ror2 regulate expression of XPAPC through an alternative noncanonical signaling pathway. Dev Cell, 2007. 12(5): p. 779-92.

368. Yamamoto, H., et al., Wnt5a modulates glycogen synthase kinase 3 to induce phosphorylation of receptor tyrosine kinase Ror2. Genes Cells, 2007. 12(11): p. 1215-23.

369. Akbarzadeh, S., et al., The deleted in brachydactyly B domain of ROR2 is required for receptor activation by recruitment of Src. PLoS One, 2008. 3(3): p. e1873.

370. Kaucka, M., et al., Post-translational modifications regulate signalling by Ror1. Acta Physiol (Oxf), 2011.

371. Frank, D.A., S. Mahajan, and J. Ritz, B lymphocytes from patients with chronic lymphocytic leukemia contain signal transducer and activator of transcription (STAT) 1 and STAT3 constitutively phosphorylated on serine residues. J Clin Invest, 1997. 100(12): p. 3140-8.

372. Li, P., et al., Stat3 activates the receptor tyrosine kinase like orphan receptor-1 gene in chronic lymphocytic leukemia cells. PLoS One, 2010. 5(7): p. e11859.

373. Aaronson, D.S. and C.M. Horvath, A road map for those who don't know JAK-STAT. Science, 2002. 296(5573): p. 1653-5.

374. Valenzuela, D.M., et al., Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. Neuron, 1995. 15(3): p. 573-84.

375. Paganoni, S., K.L. Anderson, and A. Ferreira, Differential subcellular localization of Ror tyrosine kinase receptors in cultured astrocytes. Glia, 2004. 46(4): p. 456-66.

376. Bayascas, J.R. and D.R. Alessi, Regulation of Akt/PKB Ser473 phosphorylation. Mol Cell, 2005. 18(2): p. 143-5.

377. MacKeigan, J.P., L.O. Murphy, and J. Blenis, Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. Nat Cell Biol, 2005. 7(6): p. 591-600.

378. Cheson, B.D., Monoclonal antibody therapy of chronic lymphocytic leukaemia. Best Pract Res Clin Haematol, 2010. 23(1): p. 133-43.

379. Gea-Banacloche, J.C., Rituximab-associated infections. Semin Hematol, 2010. 47(2): p. 187-98.

380. Elter, T., et al., Management of infections in patients with chronic lymphocytic leukemia treated with alemtuzumab. Ann Hematol, 2009. 88(2): p. 121-32.

381. O'Mahony, D. and M.R. Bishop, Monoclonal antibody therapy. Front Biosci, 2006. 11: p. 1620-35.

382. Carter, P.J., Potent antibody therapeutics by design. Nat Rev Immunol, 2006. 6(5): p. 343-57.

383. Rader, C., Monoclonal antibody therapy for cancer. In: Fowler DH, Medin JA, eds Experimental and Applied Immunotherapy. New York: Springer, 2011: p. pp 59–83.

384. Daneshmanesh, A.H., et al., Monoclonal antibodies against ROR1 induce apoptosis of chronic lymphocytic leukemia (CLL) cells. Manuscript.

385. Spiridon, C.I., et al., Targeting multiple Her-2 epitopes with monoclonal antibodies results in improved antigrowth activity of a human breast cancer cell line in vitro and in vivo. Clin Cancer Res, 2002. 8(6): p. 1720-30.

386. Wang, L., Y.Y. Shao, and R.T. Ballock, Carboxypeptidase Z (CPZ) links thyroid hormone and Wnt signaling pathways in growth plate chondrocytes. J Bone Miner Res, 2009. 24(2): p. 265-73.

387. Decker, T., P. Kovarik, and A. Meinke, GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. J Interferon Cytokine Res, 1997. 17(3): p. 121-34.

388. Hafizi, S. and B. Dahlback, Signalling and functional diversity within the Axl subfamily of receptor tyrosine kinases. Cytokine Growth Factor Rev, 2006. 17(4): p. 295-304.

389. Trachootham, D., et al., Effective elimination of fludarabine-resistant CLL cells by PEITC through a redox-mediated mechanism. Blood, 2008. 112(5): p. 1912-22.